Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

STRESS RESPONSE MECHANISMS IN *LISTERIA MONOCYTOGENES*

Mirjami Mattila

ACADEMIC DISSERTATION

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Supervising Professor
Professor Miia Lindström, DVM, Ph.D.
Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Supervisors
Professor Emeritus Hannu Korkeala, DVM, Ph.D., M.Soc.Sc.
Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Professor Miia Lindström, DVM, Ph.D.
Department of Food Hygiene and Environmental Health
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Reviewed by
Director, Professor Aivars Bērziņš, DVM, Ph.D.
National Institute of Food Safety, Animal Health and Environment "BIOR"
Faculty of Veterinary Medicine
Latvia University of Life Sciences and Technologies
Riga, Latvia

Professor Mati Roasto, DVM, M.Sc., Ph.D.
Department of Food Hygiene and Veterinary Public Health
Institute of Veterinary Medicine and Animal Sciences
Estonian University of Life Sciences
Tarto, Estonia

Opponent
Professor Emeritus Atte von Wright, M.Sc., Ph.D.
Institute of Public Health and Clinical Nutrition
University of Eastern Finland
Kuopio, Finland

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ABSTRACT

Listeria monocytogenes is the causative agent of serious food-borne illness, listeriosis. The ability of *L monocytogenes* to survive and proliferate over a wide range of environmental conditions allows it to overcome various food preservation and safety barriers. To be able to control the risk of *L. monocytogenes* in the entire food chain, it is important to understand the mechanisms behind the stress tolerance of this pathogen. The aim of this study was to investigate the stress response mechanisms in *L. monocytogenes* strain EGD-e.

Various cellular processes needed for growth and survival under unfavourable conditions are regulated through alternative sigma factors σ^B and σ^L. To gather deeper understanding about the role of these sigma factors at low temperatures, this study investigated the regulons of σ^B and σ^L, and the expression profile of the *ΔsigBL* double-mutant strain with a whole-genome expression analysis at 3°C and 37°C. Whole genome expression analysis revealed 198 and 86 genes positively regulated by σ^B during exponential growth at 3°C and 37°C, respectively. Altogether 29 genes were found to be under positive σ^B transcriptional regulation during exponential growth at both temperatures. At 3°C, 237 genes were detected to be under positive σ^L dependent transcriptional control, and at 37°C the number of down-regulated genes was 203. Only 47 of these genes exhibited positive σ^L transcriptional regulation at both temperatures. Of the 254 genes down-regulated in *ΔsigBL* at 3°C, 38% were detected also in *ΔsigB* or *ΔsigL*, and 62% were present only in *ΔsigBL*. At 37°C, 87% of the 139 down-regulated genes were present in either *ΔsigB* or *ΔsigL* and 13% were detected only in *ΔsigBL*. It thus appears that the growth at low temperature expands the specialized expression profile of *ΔsigBL*, but at optimal growth temperature the *ΔsigBL* expression profile resembles more the expression profiles of *ΔsigB* and *ΔsigL*. The phenotypic evaluation of the *ΔsigB*, *ΔsigL*, and *ΔsigBL* mutants revealed impaired growth in the presence of ethanol, and organic acids at low growth temperature. The growth of the *sigB* null mutant was compromised in the presence of lactic acid, acetic acid, and ethanol, while both the *sigL* and *sigBL* null mutants showed impaired growth in the presence of lactic acid, acetic acid, citric acid, and ethanol relative to the wild-type strain. The growth of the *sigL* and *sigBL* null mutants was also slower at low temperature (4°C) in defined medium relative to the parental strain. In addition, phenotypic microarray analysis exposed significant differences in growth of the wild-type strain and the *sigB*, *sigL*, and *sigBL* null mutant strains on different carbon sources and in the presence of various chemical compounds, such as antibiotics targeting cell wall, membrane, DNA, or protein synthesis. Apparently, the deletion in both *sigB* and *sigL* increases the sensitivity of the *L. monocytogenes* towards different stress conditions and chemical compounds and exposes phenotypic

characteristics absent from *sigB* or *sigL* null mutant strains. The stress response mechanisms positively regulated by both *sigB* and *sigL* might thus be more relevant than has been previously suspected. Overall, this study provided an expanded insight into σ^B and σ^L phenotypic roles and functional interactions in *L. monocytogenes*.

DEAD-box proteins are conserved RNA helicases that can be found in most living organisms. They are needed in RNA metabolism and other metabolic processes and have been linked with cold stress tolerance in *L. monocytogenes* and many other bacteria. To investigate the role of DEAD-box RNA helicase encoding genes under low growth temperature, transcriptomic and phenotypic analyses of *lmo0866, lmo1246, lmo1450,* and *lmo1722* deletion mutants were performed at 3°C, 25°C, and 37°C. The relative expression levels of *lmo0866, lmo1246, lmo1450,* and *lmo1722* were significantly higher during growth at 3°C than at 37°C and also 30 min, 3 h, and 7 h after cold shock from 37°C to 5°C. At 3°C, the growth of *Δlmo0866* and *Δlmo1722* was totally impaired, and *Δlmo1450* showed only slight growth. The minimum growth temperatures of these mutants were significantly higher compared to the wild-type strain. The results suggest that *lmo0866, lmo1450,* and *lmo1722* play an important role in the cold stress tolerance of *L. monocytogenes*.

The temperature-dependent induction of flagella is a well-characterized phenomenon in *L. monocytogenes*. However, the essentiality of increased flagellum production during growth at low temperatures is still unclear. The role of flagella synthesis and motility genes *flhA* and *motA* in the cold stress tolerance of *L. monocytogenes* was studied at 3°C, 25°C, and 37°C. The relative expression levels of *flhA* and *motA* were found to be higher at 3°C compared to both 25°C and 37°C. The growth of the *ΔflhA* and *ΔmotA* deletion mutant strains was compromised at 3°C relative to the wild-type strain. These results illustrate that *flhA* and *motA* have a role in the cold stress tolerance of *L. monocytogenes,* yet the exact functions and regulation of these genes at low growth temperatures remains unknown.

Deletion mutant *ΔsigL* was completely non-motile at 3°C and 10°C, and un-flagellated at 3°C. The *ΔsigB* and *ΔsigBL* mutants showed similar swarming pattern compared to the wild-type at 3°C, but the flagella formation of *ΔsigBL* was slightly compromised at 3°C compared to the wild-type. The cold-sensitive deletion mutant strains *Δlmo0866* and *Δlmo1450* were completely non-motile in BHI at 3°C, while the swarming pattern of the *Δlmo1246* was similar to the wild-type strain. The deletion mutant strains *ΔflhA* and *ΔmotA* were completely non-motile at 3°C and 25°C, and flagella formation was deficient. The growth of these strains was also significantly compromised when grown in BHI at 3°C. These findings suggest that the motility and flagella formation may play a role in the cold response of *L. monocytogenes*.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications referenced in the text by their Roman numerals:

I Mattila, M., Somervuo, P., Korkeala, H., Stephan, R. and Tasara, T., 2020. Transcriptomic and Phenotypic Analyses of the Sigma B-Dependent Characteristics and the Synergism between Sigma B and Sigma L in Listeria monocytogenes EGD-e. *Microorganisms*, *8*(11), p.E1644.

II Mattila, M., Somervuo, P., Rattei, T., Korkeala, H., Stephan, R. and Tasara, T., 2012. Phenotypic and transcriptomic analyses of Sigma L-dependent characteristics in Listeria monocytogenes EGD-e. *Food microbiology*, *32*(1), pp.152-164.

III Markkula, A., Mattila, M., Lindström, M. and Korkeala, H., 2012. Genes encoding putative DEAD-box RNA helicases in Listeria monocytogenes EGD-e are needed for growth and motility at 3°C. *Environmental microbiology*, *14*(8), pp.2223-2232.

IV Mattila, M., Lindström, M., Somervuo, P., Markkula, A. and Korkeala, H., 2011. Role of flhA and motA in growth of Listeria monocytogenes at low temperatures. *International journal of food microbiology*, *148*(3), pp.177-183.

ABBREVIATIONS

ATP	Adenosine triphosphate
ATR	Acid tolerance response
a_w	Water activity
BHI	Brain hearth infusion
bp	Base pair
cDNA	Complementary DNA
CDS	Coding sequence
CFU	Colony forming unit
CSP	Cold shock protein
DM	Defined medium
DNA	Deoxyribonucleic acid
GAD	Glutamate decarboxylase
GSR	General stress response
HK	Histidine kinase
MIC	Minimum inhibitory concentration
OD_{600}	Optical density at 600 nm
PCR	Polymerase chain reaction
PM	Phenotypic microarray
PTS	Phosphotransferase system
RNA	Ribonucleic acid
ROS	Reactive oxygen compounds
RR	Response regulator
RT	Reverse transcription
RTE	Ready-to-eat
RT-qPCR	Quantitative real-time reverse transcription PCR
TCS	Two-component regulatory system
tRNA	Transfer RNA
WGS	Whole genome sequencing

1 INTRODUCTION

Professor Gustav Hülphers was the first to isolate the gram-positive bacterium *Listeria monocytogenes* from a liver necrosis in rabbits in 1911 (Hülphers, 1911). He named the novel bacterium *Bacillus hepatica*. Unfortunately, this strain was not preserved. In 1924, E.G.D. Murray then isolated *L. monocytogenes* from the blood of dead laboratory rabbits and guinea pigs calling this new agent *Bacterium monoytogenes* because of the mononuclear leucocytosis observed in the animals (Murray et al., 1926). Murray, Webb, and Swann are consequently regarded as the discoverers of *L. monocytogenes*. In 1940, the genus was named *Listeria* by Pirie (1940) who isolated the bacterium in 1927 from wild gerbils in South Africa naming it first *Listerella hepatolytica* to honor Sir Joseph Lister, the discoverer of antisepsis (Pirie, 1927). In Denmark in 1929 the first human isolation of *L. monocytogenes* was confirmed by Nyfeldt (1929) but it took over 20 years before the bacterium was recognized as the causative agent of serious newborn or stillborn infants' disease—listeriosis (Reiss et al., 1951).

L. monocytogenes has been called an emerging food-borne pathogen because it was recognized only a few decades ago that *L. monocytogenes* can be transmitted via food. Already in the 1950s, German scientist Heinz P. R. Seeliger suspected that the listeriosis outbreak in Halle, East-Germany, was food-related (Seeliger, 1961). In 1980s, the importance of this bacterium came to widespread attention when several foodborne listeriosis outbreaks were described (Schlech et al., 1983; Fleming et al., 1985; Linnan et al., 1988). *L. monocytogenes* was officially identified as a cause of foodborne illness for the first time in 1981 during an outbreak in Halifax, Nova Scotia. The outbreak involved 41 cases, mostly pregnant women and neonates, and a total of 18 people died due to the illness. Consumption of coleslaw salad, which contained cabbage that had been contaminated with *L. monocytogenes* containing sheep manure, was epidemiologically linked to the outbreak (Schlech et al., 1983). Since then, *L. monocytogenes* has been widely recognized as a significant risk in the food industry and as a substantial causative agent of foodborne outbreaks.

L. monocytogenes is commonly found in different raw materials used in the food industry (Miettinen et al., 2001; Markkula et al., 2005; Ruusunen et al., 2013). The most common vehicle foods are ready-to-eat (RTE) products that have not undergone a sufficient heat treatment, or that are processed after the heat treatment and thus susceptible to cross contamination (Bērziņš et al., 2009; Lopez-Valladares et al., 2018; Koskar et al., 2019). *L. monoctogenes* is remarkably tolerant to a wide range of stress conditions, and as a consequence, this pathogen is extremely difficult to control in the entire food chain (NicAogáin & O'Byrne, 2016; Bucur et al., 2018). The stress conditions that *L. monocytogenes* encounters may be related to the food matrix, such as low

pH, or be connected to the food preservation methods, like increased salt concentrations. Some of the stress conditions the bacteria may encounter are linked to technical methods to preserve foods, such as thermal treatments or cold storage. The aim with all these procedures is to either kill the pathogenic bacteria present in the products, or to inhibit their growth during storage. The exceptional stress tolerance of *L. monocytogenes* makes it an interesting model organism to investigate the stress tolerance mechanisms of foodborne pathogens.

2 REVIEW OF THE LITERATURE

2.1 *LISTERIA MONOCYTOGENES* AND LISTERIOSIS

2.1.1 THE GENUS *LISTERIA*

The genus *Listeria* belongs to the family *Listeriaceae* together with the genus *Brochothrix* and in the class of *Bacilli* of the phylum *Firmicutes* (Rocourt & Buchrieser, 2007; Whitman et al., 2012). The genus currently contains 17 species: *L. monocytogenes, L. innocua, L. ivanovii, L. seeligeri, L. welchimeri, L. grayi, L. rocourtiae, L. marthii, L. fleischmannii, L. weistephanensis, L. floridensis, L. aquatica, L. newyorkensis, L. cornellensis, L. grandensis, L. riparia, and L. booriae* (Orsi & Wiedmann, 2016). Only two of these species (*L. monocytogenes* and *L. ivanovii*) are considered to be pathogens, and eleven (*L. marthii*, *L. rocourtiae*, *L. weihenstephanensis*, *L. grandensis*, *L. riparia*, *L. booriae*, *L. fleischmannii*, *L. floridensis*, *L. aquatica*, *L. newyorkensis*, and *L. cornellensis*) have been described since 2009 (den Bakker et al., 2010; Graves et al., 2010; Leclercq et al., 2010; Bertsch et al., 2013; Halter et al., 2013; Weller et al., 2015). Prior to this, the last time a new Listeria species had been described happened in 1984 when Seeliger et al. (1984) described *L. ivanovii*. The 17 *Listeria* species can be devided into two categories based upon their phenotypic and genotypic characteristics. The Listeria sensu strictu group includes *L. monocytogenes, L. seeligeri, L. marthii, L. ivanovii, L. welshimeri, and L. innocua*, and the Listeria sensu lato group the other 11 *Listeria* species (Chiara et al., 2015; Orsi & Wiedmann, 2016).

2.1.2 CHARACTERISTICS OF *L. MONOCYTOGENES*

L. monocytogenes is a small rod-shaped Gram-positive bacterium which is 1–2 μm long and 0.5 μm in diameter. It is catalase-positive and oxidase-negative and does not form spores or capsula. It is β-haemolytic and typically ferments rhamnose but not xylose (Seeliger & Jones, 1986; Rocourt & Buchrieser, 2007; McLauchlin & Rees, 2009). *L.monocytogenes* strains are motile and form peritrichious flagella at temperatures below 30°C. At 37°C, *L. monocytogenes* strains are virtually non-motile and produce little or no detectable flagellin (Gründling et al., 2004). A characteristic tumbling motility is observed in a hanging-drop preparation, and it produces a typical umbrella shaped growth pattern at room temperature in a stab culture in semisolid medium (Peel et al., 1988; Way et al., 2004; Rocourt & Buchrieser, 2007; Bertsch et al., 2013).

L. monocytogenes strains are divided into 13 serotypes based on O and H antigens (Seeliger & Höhne, 1979) and further into four genetic lineages.

Lineage I contains serotypes 1/2b, 3b, 3c, and 4b; lineage II contains serotypes 1/2a, 1/2c, and 3a; and lineages III and IV contain serotypes 4a, 4b, and 4c (Piffaretti et al., 1989; Wiedmann et al., 1997; Nadon et al., 2001; Ward et al., 2008; Orsi et al., 2011). Most of the *L. monocytogenes* strains belong to lineages I and II (Piffaretti et al. 1989; Doumith et al., 2004)

Glaser et al. (2001) were the first to sequence the complete genome of *L monocytogenes*. The strain EGD-e genome consists of 2,944,528 base pairs with 2867 protein-coding genes and a low average DNA GC content of 39% (Glaser et al., 2001). In August, 2020, a total of 203 completed *L. monocytogenes* genome sequences were publicly accessible in sequence databases (GenBank, EMBL, GOLD).

2.1.3 LISTERIOSIS

L. monocytogenes causes a life-threatening disease, listeriosis, that mostly affects pregnant women, new-borns, the elderly and immunocompromised, and people with other underlying diseases. *L. monocytogenes* is the main cause of listeriosis in animals, even though *L. ivanovii* is also pathogenic and causes disease mostly to ruminants (Nyfeldt, 1929; Ivanov, 1962; Vázquez-Boland et al., 2001). In rare cases, other Listeria spp. have also been shown to be able to cause similar symptoms as *L. monocytogenes* in people with predisposing factors (Rocourt et al., 1986; Perrin et al., 2003; Rapose et al., 2008; Guillet et al., 2010).

Human listeriosis

Listeriosis is a fatal infection with mortality rate of 20–30% in its invasive form (Mylonakis et al., 1998; Vázquez-Boland et al., 2001; Silk et al., 2012). Typical symptoms include fever, vomiting, diarrhoea, abdominal pain, and flu-like illness that can be very mild. Pregnant women have also reported headache, backache, and dizziness (Craig et al., 1996; Goulet et al., 2012; Vázquez-Bolandet al., 2017). In pregnant women, listeriosis can lead to septicaemia and eventually to abortion, stillbirth, or premature birth. Newborns may exhibit pneumonia, septicaemia, or meningitis (Mylonakis et al., 2002, Vázquez-Boland et al., 2017). Listeriosis can also cause meningitis and encephalitis, or affect other parts of the central nervous system. In these cases, patients might show symptoms such as neck stiffness, seizures, hemiparesis, ataxia, or even coma (Lavetter et al. 1971; Vázquez-Boland et al., 2001; Brouwer et al., 2006; Swaminathan & Gerner-Smidt, 2007).

In healthy adults *L. monocytogenes* can cause gastroenteritis and the invasive form of the disease is rare. The most typical symptoms are fever, non-bloody diarrhea, headache, nausea, arthralgia, and abdominal pain. Children usually have fever and vomiting, but more rarely diarrhea and arthralgia. Severe gastroenteritis might also lead to hospitalization, especially in children and the elderly (Miettinen et al., 1999b; Aureli et al., 2000; Frye et al., 2002; Ooi and Lorber, 2005; Goulet et al., 2012). *L. monocytogenes* can also rarely

cause eye and cutaneous infections, mostly in people having direct contact with infected material such as infected foetuses, like veterinarians or farmers (McLauchlin & Low, 1994; Betriu et al., 2001; Regan et al., 2005; Tay et al., 2008; Godshall et al., 2013). Healthy adults may also be asymptomatic carriers of *L. monocytogenes*. The prevalence of carriers is usually low, about 1% or less (MacGowan et al., 1994; Iida et al., 1998).

The incubation period of listeriosis has been reported to vary between 1 and 70 days, where pregnant patients usually show the longest incubation times, on average 25 days, and non-pregnant patients on average 5 days (Linnan et al., 1988; Mead et al., 2006; Goulet et al., 2013). The incubation period of non-invasive gastroenteritis can vary from 6 hours to 10 days, but the symptoms typically begin within 24 hours after ingestion of the bacterium, and can last 1–3 days or even up to one week (Miettinen et al., 1999b; Aureli et al., 2000; Vázquez-Boland et al., 2001; Ooi & Lorber, 2005).

Development of clinical listeriosis seems to be dose-dependent (Miettinen et al., 1999b; Maijala et al., 2001; Ooi & Lorber, 2005). Prolonged consumption of contaminated food increases the risk of infection, and a daily intake of as few as 10^3 cfu of *L. monocytogenes* might already be a sufficient dose to infect people belonging to the risk groups (Ericsson et al., 1997; Maijala et al., 2001). The infectious dose for non-invasive gastroenteritis in non-susceptiple people has been reported to be 10^5 cfu/g or higher (Dalton et al., 1997; Miettinen et al., 1999b; Aureli et al., 2000; Ooi & Lorber, 2005).

When the bacteria enter the stomach, gastric acids reduce the number of viable bacteria, and the cells that have survived the low pH move forward to small intestine (Schlech et al., 1993; Vázquez-Boland et al., 2001). The bacterial cells are able to pass through the intestinal barrier through junctions of mucus-secreting goblet cells and enterocytes, through the tip of the intestinal villi with apoptotic cell extrusion or by penetrating the M cells in Peyer's patches (Ribet & Cossart, 2015; Radoshevich & Cossart, 2018). The bacterium then enters its target organs, the liver and spleen, via the lymph and blood. It can also cross the blood-brain barrier in susceptiple people and is able to penetrate the fetoplacental barrier in pregnant women, leading to clinical invasive listeriosis (Pron et al., 1998; Vázquez-Boland et al., 2001; Cossart, 2011; Melton-Witt et al., 2012). In non-susceptible people, the immune system is usually able to destroy *L. monocytogenes* in Kupffer cells in the liver (Cheers et al., 1978; Mielke et al., 1988; Gregory & Liu, 2000).

L. monocytogenes is a facultative intracellular pathogen (Kuhn et al., 1988; Portnoy, 1992; Rouquette & Berche, 1996). The pathogenicity of *L. monocytogenes* and the ability of this bacterium to penetrate and proliferate inside the host cells is due to the expression of several essential virulence genes which are clustered in *Listeria* pathogenicity islands (LIPIs) (Gonzalez-Zorn et al., 2000; Vázquez-Boland, 2001; Cotter et al., 2008; Clayton et al., 2014). *L. monocytogenes* is able to enter both phagocytic and non-phagocytic cells. The entry into non-phagocytic cells happens through receptor-mediated endocytosis. Internalin A (InlA) and InlB bind to their

corresponding receptors on host cell surface and, together, mediate bacterial invasion into host cells (Vázquez-Boland, 2001; Cossart, 2011; Cossart & Helenius, 2014).

Foodborne outbreaks

Almost all human listeriosis cases (99%) are food related (Schlech et al., 1983; Pinner et al., 1992; Mead et al., 2006), even though some atypical cases of direct transmission from animals to humans has been reported (McLauchlin & Low, 1994; Regan et al., 2005). Many cases are sporadic since the long incubation period of the disease makes epidemiological investigation difficult, and the vehicle food often remains unrecognized. However, several listeriosis outbreaks have been reported all over the world (Fleming et al., 1985; Linnan et al., 1988; Lyytikäinen et al., 2000; Gaul et al., 2013; Lomonaco et al., 2013; Magalhães et al., 2015; Coroneo et al., 2016; Kvistholm et al., 2016; Self et al., 2016; Angelo et al., 2017; Dahl et al., 2017; Schjørring et al., 2017; Allam et al., 2018). Selected foodborne outbreaks worldwide during 2009–2020 caused by *L. monocyogenes* are presented in Table 1.

Table 1. Selected foodborne outbreaks worldwide 2010-–2020 caused by *Listeria monocyogenes*

Country	Year	Food type	No. of cases	Deaths	Serotype	References
Portugal	2009–12	Cheese	30	11	4b	Magalhães et al., 2015
USA	2010	Pre-cut celery	10	5	1/2a	Gaul et al., 2013
USA	2011	Cantaloupe	147	33	1/2a, 1/2b	Lomonaco et al., 2013
USA	2012	Ricotta Salata Cheese	22	4	NR	Coroneo et al., 2016
Denmark	2013–14	Pork sausage	41	17	1/2b	Kvistholm et al., 2016
Sweden	2013–14	Meat cold-cuts	51	NR	1/2a	Dahl et al., 2017
USA	2014	Caramel apples	35	7	4b	Angelo et al., 2017
USA	2015	Soft cheese	30	3	NR	CDC report, 2015
EU	2015–18	Frozen corn	32	6	4b	EFSA report, 2018
USA	2016	Packaged salads	19	1	NR	Self et al., 2016
South Africa	2017–18	“Polony” meat	1060	216	4b	Smith et al., 2019
USA	2019	Deli-Sliced Meats and Cheeses	10	1	NR	CDC report, 2019
USA	2020	Enoki Mushrooms	36	4	NR	CDC report, 2020

Derived from Buchannan et al., 2017; Kaptchouang Tchatchouang et al., 2020

NR, Serotype not recorded

Listeriosis outbreaks have been linked mostly to RTE meat, fish and dairy products (Gaul et al., 2013; Little et al., 2012; Dahl et al., 2017; Mäesaar & Roasto, 2020) but during recent years some novel plant-based vehicle foods have also been recognized. In 2011, contaminated cantaloupe melon caused one of the largest listeriosis outbreaks in history, infecting 147 people (Lomonaco et al., 2013), and in 2014 caramel apples were recognized to be the vehicle food during the outbreak (Glass et al., 2015; Angelo et al., 2017). Frozen corn originating from Hungary caused an outbreak in the EU in 2015–2018, and altogether 32 people were infected in the outbreak in five EU member states (Austria, Denmark, Finland, Sweden, and the United Kingdom) (EFSA report, 2018).

The largest known listeriosis outbreak to date happened in South Africa in 2017–2018 (Smith et al., 2019; Kaptchouang Tchatchouang et al., 2020). A total of 1060 cases were reported with a mortality rate of 20%. Epidemiological investigation together with whole genome sequencing (WGS) revealed that ready-to-eat processed meat products (chicken polony) were the source of the outbreak.

L. monocytogenes serotypes most commonly found in food samples belong to serogroup 1/2, and the serotypes most often causing clinical listeriosis are 1/2a, 1/2b, and 4b (Gilot et al., 1996; Aouaj et al., 2002; Bērziņš et al., 2009; Gianfranceschi et al., 2009; Braga et al., 2017; Chen et al., 2020). In Europe, and especially in Nordic countries, serotype 1/2a is commonly seen in clinical cases (Lukinmaa et al., 2003; Parihar et al., 2008; Lopez-Valladres et al., 2014; Lopez-Valladares et al., 2018) while serotype 4b has often been connected with severe listeriosis cases in the USA (Pinner et al., 1992; Cartwright et al., 2013).

The annual incidence of human listeriosis cases is approximately 0.5 cases per 100,000 population in EU/EEA countries and 0.3 per 100,000 in the USA (Silk et al., 2012; ECDC Annual Epidemiological Report, 2017; EFSA and ECDC report, 2019; Pohl et al., 2019). During the years 2009–2018, a statistically significant increasing trend of confirmed listeriosis cases has been observed in the EU. During the same 10-year period a clear seasonal pattern was observed with a higher number of listeriosis cases during summer, followed by a smaller number of cases during the winter season (EFSA and ECDC report, 2019).

In 2018, EU member states reported 2549 confirmed cases of invasive human listeriosis whereas in 2017 the number of confirmed cases was 2502. The number of confirmed listeriosis cases and rates per 100,000 people in the EU and Nordic countries during 2014–2018 are presented in Table 2. The figures show that Finland, together with other Nordic countries, has had one of the highest notification rates every year during the five-year observation period. The mean annual rate in Finland has been 1.26 per 100,000 people whereas the mean rate in whole EU has been 0.46 during the same period (EFSA and ECDC report, 2019).

Table 2. The number of confirmed listeriosis cases and rates per 100,000 people in Nordic countries and in EU, 2014–2018

Country	2014		2015		2016		2017		2018	
	No.	Rate	No.	Rate	No.	Rate	No.	Rate	No.	Rate
Finland	65	1.19	46	0.84	67	1.22	89	1.62	80	1.45
Sweden	125	1.30	88	0.90	68	0.69	81	0.81	89	0.88
Denmark	92	1.64	44	0.78	40	0.70	58	1.01	49	0.85
Norway	29	0.57	18	0.35	19	0.37	16	0.30	24	0.45
Iceland	4	1.23	0	0	0	0	6	1.77	2	0.57
EU	2217	0.46	2183	0.43	2503	0.47	2479	0.48	2549	0.47

Derived from ECDC Annual Epidemiological Report, 2017 and EFSA and ECDC report, 2019

Listeriosis in animals

L. monocytogenes was first described as animal pathogen in 1926 when Murray et al. (1926) found the bacterium in the livers of sick guinea pigs and rabbits. Listeriosis was later also described in various species, both mammals and birds, as a cause of zoonosis (Gray & Killinger, 1966; Low & Donachie, 1997; Nightingale et al., 2004; Lecuit, 2007). Regardless of the ability of *L. monocytogenes* to infect several different animal species, listeriosis is primarily regarded as a disease of ruminants. In contrast to human listeriosis, *L. ivanovii* can also cause a clinical disease to animals (Ivanov, 1962; Gray & Killinger, 1966; Gouin et al., 1994; Khelef et al., 2006).

Clinical listeriosis is most often seen in sheep, but cows and goats have also been reported to suffer from the disease (Beauregard & Malkin, 1971; Low & Donachie, 1997; Chand & Sadana, 1999; Wiedmann et al., 1999; Wagner et al., 2005; Lecuit, 2007; Bundrant et al., 2011). In adult ruminants, encephalitis is the most common clinical manifestation, showing symptoms such as ataxia, going in circles, opistotonus, anorexia, and depression. Abortions and stillbirths happen especially during the third semester of gestation, and the infected animals can also show symptoms such as conjunctivitis, mastitis, gastroenteritis, hepatitis, or pneumonia. Septicemia is mostly seen in neonates and young animals (Jensen et al., 1996; Clark et al., 2004; Lecuit, 2007; Rawool et al., 2007; Ryser & Marth, 2007; Brugère-Picoux, 2008).

In ruminants, ingestion of contaminated feed, especially spoiled silage has been associated with listeriosis. Other predisposing factors are indoor housing and poor management practices, and the highest incidence is seen during winter and early spring (Wilesmith & Gitter, 1986; Fenlon et al., 1989; Husu et al., 1990a; Sanaa et al., 1993, Nightingale et al., 2005).

Listeriosis is seldom seen in most monogastric mammals like in horses, pigs, cats, or dogs. On the other hand, rodents like chinchillas, as well as rabbits, seems to be susceptible to infection (Finley & Long, 1977; Wilkerson

et al., 1997; Wesley, 2007; Wesley et al., 2008; Hoelzer et al., 2012). In birds, listeriosis is rare and is often a secondary infection related to viral, bacterial, parasitic, or tumor disease (Cummins et al., 1988; Wesley, 2007). Bacterial shedding without clinical symptoms has been reported both in mammals and in birds (Husu, 1990; Iida et al., 1998; Unnerstad et al., 2000; Nightingale et al., 2004; Ho et al., 2007; Lyautey et al., 2007; Hellström et al., 2008; Esteban et al., 2009).

2.2 *L. MONOCYTOGENES* IN FOOD CHAIN

Listeria species are ubiquitous bacteria widely present in plant and soil. *L. monocytogenes* has been isolated for example from cattle, sheep, goats, and poultry, but uncommonly from wild animals (Gray & Killinger, 1966). *L. monocytogenes* has also been found in water supplies, sewage, silage, fish farms, slaughterhouse waste, milk, and human and animal feces (Weis & Seeliger, 1975; Husu, 1990; Husu et al., 1990a; Husu et al., 1990b; MacGowan et al., 1994; Pauly and Tham, 2003; Katzav et al., 2006; Miettinen & Wirtanen, 2006). These specific characteristics of the bacteria lead inevitably to presence of *L. monocytogenes* in the food chain resulting in contamination of various types of food products.

2.2.1 *L. MONOCYTOGENES* IN FOODS

The importance of *L. monocytogenes* as a foodborne pathogen was markedly increased in the 1980s after several food-borne outbreaks (Schlech et al., 1983; Fleming et al., 1985; Linnan et al., 1988). Since then, several outbreaks and sporadic cases related to *L. monocytogenes* have been reported (Farber & Peterkin, 1991; Miettinen et al., 1999b; Lyytikäinen et al., 2000; Lomonaco et al., 2013; Magalhães et al., 2015).

L. monocytogenes has been isolated from different types of raw food materials, especially in poultry, pork, and fish (Autio et al., 1999; Autio et al., 2000; Miettinen et al., 2001; Markkula et al., 2005; Miettinen & Wirtanen, 2005). Listeria spp. and *L. monocytogenes* has also been isolated in bulk tank-milk and in raw milk samples (Husu et al., 1990b; Moura et al., 1993; Waak et al., 2002; Kells & Gilmour, 2004; Ruusunen et al., 2013, Kalmus et al., 2015; Castro et al., 2017).

Several studies have linked ready-to-eat (RTE) foods to listeriosis and RTE foods are currently considered to be the most important vehicles for human infections. The most typically contaminated RTE foods associated with human listeriosis have been reported to be meat, fish, and milk products as well as foods with plant origin and frozen foods. Depending on the preparation and storage conditions of the products, nearly all types of RTE foods may promote growth of *L. monocytogenes* and thus infect people belonging to risk groups (Bērziņš et al., 2009; EFSA report, 2018; Kurpas et al., 2018; Lopez-Valladares

et al., 2018; Koskar et al., 2019; EFSA Panel on Biological Hazards (BIOHAZ), 2020).

A common factor for all of these foods is that they have not undergone a sufficient heat treatment that is able to eliminate pathogenic bacteria in the products. RTE foods are often stored at low temperatures and are highly susceptible to contamination with *L. monocytogenes,* since the bacterium is able to survive and grow at refrigerator temperatures and in the presence of many food preservation factors such as low pH and high salt concentrations (Ryan et al. 2008)

In a 2010–2011 EU-wide baseline survey on the prevalence of *L. monocytogenes* in certain RTE foods, the prevalence of *L. monocytogenes* in retail fish samples across the entire EU was 10.4%, at the time of sampling and 10.3% at the end of shelf-life. For meat and cheese samples, the prevalence at the end of shelf-life was 2.07% and 0.47%, respectively (EFSA report, 2013).

2.2.2 *L. MONOCYTOGENES* IN FOOD PROCESSING

L. monocytogenes is able to attach to different types of surfaces in the food processing premises, and to form biofilms. The biofilm protects the bacterial cells against various types of antimicrobial actions during sanitation processes. The biofilm also helps the bacteria to colonize food processing equipment and other surfaces such as floors, pipes, and drainage systems, and to persist in the food manufacturing premises. Several studies have reported *L. monocytogenes* strains that have persisted in food-processing plants, even for years (Rørvik et al., 1995; Miettinen et al., 1999a; Keto-Timonen et al., 2007).

The ability to form biofilms on various types of food-contacting surfaces makes it very difficult to control this pathogen efficiently in the food-processing environment (Kocot & Olszewska, 2017). Insufficient cleaning routines and poor hygiene practices can enhance the formation of biofilms on food contact surfaces. Biofilms and also *L. monocytogenes* itself are able to resist many types of sanitizing and disinfecting agents that are commonly used in food manufacturing establishments and thus a cross-contamination by *L. monocytogenes* is a serious and relevant food safety risk in all types of premises used for processing food (Lundén et al., 2003a; Kornacki & Gurtler, 2007; Carpentier & Cerf, 2011; Aalto-Araneda et al., 2019).

Many studies have revealed that the strains contaminating the final products are usually not the same that are found in raw materials but originating from other sources, often from the processing environment (Rørvik et al., 1995; Autio et al., 1999; Giovannacci et al., 1999). Several different processes and different types of processing machines, such as slicing, cutting, and brining machines and conveyer belts, have been associated with *L. monocytogenes* contamination in food manufacturing premises (Autio et al., 1999; Miettinen et al., 1999b; Lundén et al., 2003b; Bērziņš et al., 2007; Keto-Timonen et al., 2007; Bērziņš et al., 2010; Aalto-Araneda et al., 2019).

Contamination of products happens usually during the processes coming after the heating treatment thus making the control of *L. monocytogenes* especially demanding, particularly regarding to RTE products (Tompkin, 2002; Reij et al., 2004).

2.3 STRESS TOLERANCE MECHANISMS OF *L. MONOCYTOGENES*

L. monocytogenes is a facultatively anaerobic, psycrotrophic, and mesophilic bacterium that can grow and survive over a wide range of temperature, pH, and water activity (a_w) (Table 3). Although *L. monocytogenes* persists in various environmental conditions, it is relatively sensitive to high temperature; heating for 30 min to 60°C kills the bacterium (Gray & Killinger, 1966; McLaughlin & Rees, 2009). *L.* mo*nocytogenes* grows well in aerobic conditions but restricted oxygen level enhances its growth (Buchanan & Klawitter, 1990). The capacity of this bacterium to grow at low temperatures as well as under osmotic, pH, and oxidative stress varies substantially between different strains (Ribeiro et al., 2006; Byelashov et al., 2009; Aalto-Araneda et al., 2020; Manso et al., 2020). This has opened an interesting field of study related to the identification of the strains of *L. monocytogenes* with different growth capacities, as well as to investigate the molecular mechanisms which help the bacteria to survive in challenging environmental conditions.

Table 3. The limits of growth for *Listeria monocytogenes*

Growth conditions	Minimum	Optimum	Maximum	References
Temperature (°C)	-1.5	37	45	Gray & Killinger, 1966; Junttila et al., 1988; Petran et al., 1989; Walker et al., 1990; Hudson et al., 1994
pH	4.4	7.0	9.6	Gray & Killinger, 1966; George et al., 1988; Farber et al.,1989; Phan-Thanh et al., 2000
Salt (%)	NR	NR	10	McClure et al., 1989; Tienungoon et al., 2000; Shabala et al., 2008
Water activity (a_w)	0.90	≥0.97	NR	Petran et al., 1989, Farber et al. 1992; Nolan et al.,1992; Tienungoon et al., 2000

NR=Not relevant

Due to a large variability in stress tolerance between different strains, *L. monocytogenes* is extremely difficult to control in the entire food chain (Aalto-Araneda et al., 2020). To be able to control the risk of proliferation of *L. monocytogenes* from production to storage and consumption of foods it is important to understand the mechanisms behind the stress tolerance of this pathogen. During the past years, the demand from consumers of more natural

and minimally processed food products has led to testing of alternative treatments and technologies, such as UV light, high pressure and the use of bacteriocins for food preservation. More knowledge is still needed to understand the efficacy and combination of these methods and to find the best techniques and solutions regarding both food safety and nutritional and sensory properties of products.

2.3.1 FOOD PRESERVATION METHODS AND STRESS TOLERANCE

Low temperature stress

Cold chain and storage of foods at low temperature is a common method to increase the shelf life of perishable foodstuff. As a psychrotrophic bacterium, the ability of *L. monocytogenes* to survive and grow is as low as -1.5°C making it an extremely difficult pathogen to control in refrigerated food products (Hudson et al, 1994; Tasara & Stephan, 2006; Chan & Wiedmann, 2008).

Exposure to cold affects bacterial cells in several ways and a sudden drop in temperature leads to a general slowdown of various cellular processes. Sudden temperature drop causes a decrease in cell-membrane fluidity, which may affect membrane structure and membrane–associated functions. RNA and DNA secondary structures start to alter, and ribosomes and other cellular macromolecules start to destabilize (Phadtare, 2004; Tasara & Stephan, 2006; Dahlsten et al., 2014).

L. monocytogenes has been shown to resist cold by maintaining cellular membrane fluidity through reducing fatty-acid chain length, increasing the concentration of unsaturated fatty acids, and altering ratios of iso- and anteiso-branched fatty acids (Püttmann et al., 1993; Russell et al., 1995; Annous et al., 1997; Beales, 2004; Neunlist et al., 2005). Another mechanism to withstand low temperatures is the import of cryoprotectative osmolytes, like glycine betaine and carnitine, from the environment into the cell through three compatible solute systems; glycine betaine porter I (BetL), glycine betaine porter II (Gbu), and the carnitine transporter OpuC (Ko et al, 1994; Verheul et al., 1997a; Angelidis & Smith, 2003; Wemekamp-Kamphuis et al., 2004a). *L. monocytogenes* can also endure cold by means of stabilization of ribosomal structure and through the production of cold shock proteins (CSPs) (Bayles et al., 2000; Wemekamp-Kamphuis et al., 2002; Tasara & Stephan, 2006; Chan et al., 2007b; Schmid et al., 2009). CSPs are small proteins, approximately 7 kDa, that many bacteria produce as a response to rapid temperature downshift. CSPs are nucleic acid binding proteins that can act as RNA chaperones. They facilitate the efficient transcription and translation within bacterial cells at low temperatures by destabilizing secondary structures in target RNA (Phadtare et al., 1999; Hebraud & Guzzo, 2000; Ermolenko & Makhatadze, 2002; Keto-Timonen et al., 2016).

Heat stress

Heating is one of the key processes used by the food industry to destroy foodborne pathogens and to ensure the safety of products. *L. monocytogenes* does not tolerate high temperatures well and regular pasteurization processes are able to reduce the number of *L. monocytogenes* occurring in the food. Nevertheless, the bacterium has been reported to survive mild or brief thermal treatments (Doyle et al, 1987, 2001). Even though *L. monocytogenes* is not particularly resistant to high temperatures, it has been shown to tolerate heat better than many other non-spore-forming pathogens (Abdel & Mattar, 2001; Huang, 2004; Sallami et al., 2006).

Heat resistance of *L. monocytogenes* varies between strains and is affected by several factors such as test and growth conditions, previous growth conditions or exposure to environmental stresses, and composition of the heating medium (Doyle et al., 2001). Resistance to heat varies considerably between different strains and serotypes (Sörqvist et al., 1994; Lundén et al., 2008). Serotype 1/2a strains seem to have a generally lower tolerance to heat while serotypes 1/2b and 4b strains seem to better tolerate high temperatures (Shen et al., 2014). Interestingly, exposure to sublethal stresses before heat treatment has been shown to increase the thermal resistance of *L. monocytogenes*. Mild, short-term heat treatment, or exposure to salt or to low pH prior to heating, have all been reported to potentiate the ability of *L. monocytogenes* to tolerate thermal treatments (Jørgensen et al., 1995, 1999; Mazzotta, 2001; Shen at al., 2014)

Exposure to high temperature induces several reactions on the cellular level. DNA and RNA start to degrade, proteins start to denaturate, and the cytoplasmic membrane is damaged, leading to leakage of cellular components. As a response, bacteria try to restore nucleic acid and protein functions and repair damaged cell-membranes (Russell, 2003; van der Veen et al., 2007; Soni et al., 2011). At the molecular level, the heat-shock response of *L. monocytogenes* comprises of the expression of genes belonging to specific heat-shock regulons (class I and class III heat-shock genes) and genes of the SigB-regulated class II general stress response. The role of heat-shock proteins is to stabilize new proteins to ensure correct folding, and to prevent their aggregation under stress conditions. Activation of SOS response, associated with DNA damage and repair, has also been described (van der Veen et al., 2007, 2009).

Acid and alkaline stress

Acidification has been widely used for centuries to preserve different kinds of foods, both from animal and plant origin. The preserving effect of fermentation is based on the drop of pH during the process and on the production of weak organic acids, like lactate and acetate. These acids have antibacterial properties and can be used as preservatives in food production (Caplice and Fitzgerald, 1999; Ross et al., 2002). Many foods, like fruits, are

also naturally acidic. On the other hand, many sanitizing agents and disinfectants used in the food industry are alkaline (Krysinski et al., 1992).

L. monocytogenes encounters acid stress both in low pH foods and while infecting the host species. Acid stress disturbs enzymatic functions and cellular bioenergetics, and damages cell membrane and DNA (Cotter & Hill, 2003; Krulwich et al., 2011; Soni et al., 2011; Smith et al., 2013).

When *L. monocytogenes* is exposed to low pH, an acid tolerance response (ATR) is observed, meaning that the microbial cells become more resistant to lethal effects of acids after an exposure to milder acid stress (Davis et al., 1996). *L. monocytogenes* retains several mechanisms to maintain cytoplasmic pH under acid stress. It can decrease the membrane permeability to protons through changes in the cell membrane, buffer the cytoplasm, and balance the external pH through catabolism (Phan-Thanh & Jansch, 2006). While exposed to acidic conditions *L. monocytogenes* also starts to produce acid stress response proteins (ASPs) such as cell respiration-related proteins. ASPs also play a role in osmolyte transport, protein folding and repair, flagella synthesis, and metabolism (Phan-Thanh & Mahouin, 1999; Phan-Thanh & Jansch, 2006; Ryan et al., 2008).

L. monocytogenes is relatively resistant to alkaline stress when compared to other food-related pathogens such as Salmonella and *Escherichia coli* (Mendonca et al., 1994). Exposure of bacteria to alkali stress disturbes enzymatic functions and cellular bioenergetics, and damages cell membrane leading to leakage of intracellular contents. Disturbances in cell division has also been reported (Giotis et al., 2007; Krulwich et al., 2011). Alkali stress induces the synthesis of heat shock proteins DnaK and GroEL (Giotis et al., 2008b), and expression of putative transporter genes (Gardan et al., 2003).

Osmotic stress

Salts and sugars are used in food production to increase the shelf life of different foodstuff, especially conserved products, as well as to give them their preferred taste and flavor. Adding these substances to foods affects the water activity and induces osmotic stress to bacteria present in the products (Burgess et al., 2016).

L. monocytogenes is able to grow and survive at high salt levels and low water activity. The bacterium tolerates salt concentrations, even up to 25% of NaCl and may survive in such environments for extended periods (Shabala et al., 2008; Boyer et al., 2009; Tiganitas et al., 2009; Schirmer et al., 2014). The ability to tolerate high salt concentrations varies between different strains and serotypes. Aalto-Araneda et al. (2020) showed that *L. monocytogenes* lineage I strains (serovars 4b and 1/2b) were significantly more tolerant toward 9.0% NaCl than lineage II strains (serovars 1/2a, 1/2c, and 3a).

L. monocytogenes tries to reduce osmotic pressure and water loss by accumulation of compatible solutes, such as carnitine and glycine betaine, in the cytoplasm. They have also shown to be able to stabilize enzyme structure and function under osmotic stress (Lippert & Galinski, 1992; Duché et al.,

2002; Bae et al., 2012). Another mechanism to combat osmotic stress is the expression of genes coding CSPs, which are also needed in the cold stress response of *L. monocytogenes*. The activity of these proteins is assumed to facilitate the repair of DNA lesions induced by high NaCl concentrations (Dmitrieva et al., 2004; Schmid et al., 2009).

The gene expression patterns under osmotic and cold stress are similar in relation to transporter systems and to cold shock proteins, suggesting that *L. monocytogenes* might counteract osmotic and cold stress conditions via similar mechasims (Smith, 1996; Mendum & Smith, 2002; Wemekamp-Kamphuis et al., 2004b; Schmid et al., 2009; Soni et al., 2011).

Oxidative stress

Bacteria encounter oxidative stress in food production due to the use of hydrogen peroxide (H_2O_2) containing sanitizers and disinfectants. *L. monocytogenes* needs to battle oxidative stress also due to atmospheric modification in the food environment as well as during host infection (Pereira et al., 2018; Yun et al., 2012).

Under oxidative stress, bacterial cells encounter high concentrations of oxygen radicals. This disturbs the redox balance of bacterial cells and leads eventually to death of the cell (Suo et al., 2014). Oxidative stress causes several types of oxidative damage to proteins, lipids, enzymes, nucleic acids, and cell membrane (Imlay et al., 2013; Ezraty et al., 2017). To cope against oxidative stress, bacteria need to detoxify reactive oxygen compounds (ROS) and to activate mechanisms to repair protein, membrane, and nucleic acid damages (Dröge, 2003; Archambaud et al., 2006).

L. monocytogenes activates several mechanisms to reduce oxidative damage such as expression of genes related to the oxidative response, notably oxidation-resistance gene *kat* that acts synergistically with superoxide dismutase coding gene *sod*. The expression of *sigB*, cold and heat shock proteins, and proteases clpC, clpP and groEL has also been recorded under oxidative stress (Ferreira et al., 2001; Archambaud et al., 2006; Oliver et al., 2010; Soni et al., 2011; Markkula et al., 2012; Suo et al., 2012; Manso et al., 2020).

Other food-related stress conditions

Ethanol is used in the food industry both as a sanitizer and as a preservative (Seiler & Russel, 1991). If ethanol is used as a sublethal dose, bacterial cells can experience various types of stress. Ethanol increases the permeability of cell membranes, denatures proteins both on the cell membrane and in cytoplasm, and inhibits protein folding interactions of macromolecules (Ingram, 1990; Barker & Park, 2001; Huffer et al., 2011). RNA helicase Lmo0866 also contributes to ethanol stress tolerance of *L. monocytogenes* EGD-e (Markkula et al., 2012).

Lactic acid bacteria produce antimicrobial peptides, bacteriocins, which are used as food additives for example for fruits, vegetables, and dairy and

meat products (Delves-Broughton et al., 1996; Johnson et al., 2018; Silva et al., 2018). Bacteriocins are effective against gram-positive bacteria and they kill their specific target organisms by growth inhibition, disruption of membrane homeostasis, and by forming pores in the cell membrane leading to permeabilization (Cotter et al., 2013; Zhang & Gallo, 2016; Chikindas et al., 2018). Nisin is by far the only approved bacteriocin for preserving food. It is effective against many gram-positive bacteria and is used to inhibit the growth of possible food-borne pathogens, including *L. monocytogenes* and *Clostridium botulinum*, in meat and dairy products (Gharsallaoui et al., 2016). *L. monocytogenes* tries to resist the effects of nisin by altering the composition of cytoplasmic membrane with a reduction in the content of phospholipids and an increase in the proportion of straight-chain fatty acids, aiming to prevent nisin to cross the cell membrane (Ming & Daeschel, 1993, 1995; Verheul et al., 1997b).

High pressure processing (HPP) is used as an alternative to heat treatment in the food industry. The pressure applied usually varies between 250 and 700 MPa. The bacterial cells that survive the treatment show morphological and physiological changes that may be reversible. Cells exposed to HPP show alteration in protein structure and function, increased cell membrane permeability and inhibition of metabolism, replication, and transcription (Huang et al., 2014). It has been shown that resistance of *L. monocytogenes* to HPP varies between the strains, and between different types of food matrices (Van Boeijen et al., 2008; Hereu et al., 2012; Tomasula et al., 2014).

UV-light is one of the new, alternative food decontamination methods, and it is used to kill bacteria on different surfaces, for example on the slices of RTE-products or as disinfection procedure in food processing environments (Bintsis et al., 2000; Gómez-López et al., 2007). DNA is considered the major target of UV radiation, even though it is likely that damage to other biomolecules contributes to the inhibitory effects of UV radiation. (Santos et al., 2013). The efficacy of this method depends on several factors, such as the food matrix, distance to the food product, level of contamination, and energy level given by number and frequency of the light pulses (Ozer and Demirci, 2006; Gómez-López et al., 2007; Delorme et al., 2020). *L. monocytogenes* has been reported to be more resistant to UV-light than many other pathogens (Beauchamp & Lacroix, 2012).

Pulsed electric field processing (PEF) is also an alternative to thermal treatment and is mainly used in liquid foodstuff. The short, intense electric-field pulses applied to the product inactivate the micro-organisms by destabilizing the cells and, depending on the strength of the treatment, damaging the cell membrane by forming micropores, causing leakage of cytoplasmic content (Góngora-Nieto et al., 2002). The efficacy of this method is not as much effected by the food matrix as, for example, the UV-light method (Toepfl et al., 2007). The usefulness of this method to control *L. monocytogenes* in food production is nevertheless questionable since *L. monocytogenes,* as well as other the gram-positive bacteria, seem to be

relatively tolerant to PEF (Lado and Yousef, 2002; Mosqueda-Melgar et al., 2007).

2.3.2 STRESS ADAPTATION AND CROSS PROTECTION

In food manufacturing, different factors to control bacteria are usually used following a hurdle mechanism. Lou & Yousef (1997) described a "stress hardening" phenomenon in *L. monocytogenes*, showing that a sublethal ethanol, acid, oxidative, osmotic or heat shock stress increased the resistance to other stress factors. This phenomenon should be taken into account when current food processing technologies are modified, or new ones are developed. (Lou & Yousef, 1997; Roberts et al., 2020).

Several studies have shown that exposing *L. monocytogenes* to sublethal stress makes the cells more tolerant to the same or other stresses (Lundén et al., 2003a). Sublethal acid stress has been shown to increase the acid, heat and osmotic stress tolerance in *L. monocytogenes* (Gahan et al., 1996) whereas sublethal alkali stress seems to protect the cells against thermal stress (Taormina and Beuchat, 2002). Low temperature has also been shown to protect *L. monocytogenes* against oxidative stress (Manso et al., 2020) as well as against high salt concentrations (Schmid et al., 2009). Osmotic stress can also lead to cross-protection against heat, ethanol, acid, alkali, and oxidative stress (Melo et al., 2015). Cold, acid, and osmotic stresses have also been shown to increase the antibiotic resistance of *L. monocytogenes* (Al-Nabulsi et al., 2015).

L. monocytogenes may use similar stress mechanisms for survival under different stress conditions. For example different cold shock proteins are induced and osmolyte transport systems are activated both in survival under cold stress and osmotic stress (Schmid et al, 2009), and heat shock protein related genes are expressed under heat stress, osmotic stress, and acid stress (Nair et al., 2000; Wilson et al., 2006; van der Veen et al., 2007).

Cross-protection mechanisms can be, at least partially, explained by the function of the two-component systems—*liaRS, lisRK, cesRK, agrCA*, and *virRS*—which play a role in the stress reponse of *L. monocytogenes* (Kang et al., 2015; Pöntinen et al., 2015, 2017b), and also by the function of alternative sigma factor SigB that controls the general stress response of this pathogen (Kazmierczak et al., 2003; Chaturongakul & Boor, 2006; Abram et al., 2008).

2.3.3 GENETIC FACTORS ASSOCIATED WITH STRESS RESPONSE OF *L. MONOCYTOGENES*

Sensing and responding to environmental stress factors is critical for the adaptability and survival of *L. monocytogenes*. To be able to survive in a changing environment, *L. monocytogenes* regulates the expression of stress related genes by transcriptional regulators, including alternative sigma (σ) factors, transcriptional activators, and repressors (Guariglia-Oropeza et al., 2014). The stressome is a supramolecular multi-protein complex, which acts as a stress-sensing center, and is responsible for coordinating the activation of a signal transduction pathway resulting in the activation of the alternative sigma factor sigma B (σ^B) that regulates gene transcription to provide homeostatic and protective functions to help the bacteria to survive under the stress. Stress resistance in *L. monocytogenes* can be partially explained by the presence of the general stress response (GSR), a transcriptional response which is under the control of σ^B (NicAogáin & O'Byrne, 2016; Guerreiro et al., 2020).

The regulation of stress responses in *L. monocytogenes* also involves other factors such as two-component regulatory systems (TCS) that allow the bacteria to sense and respond to changes in environmental conditions through signal transduction (Cotter et al., 1999; Kallipolitis & Ingmer, 2001; Kallipolitis et al., 2003; Pöntinen et al., 2015, 2017b). Other transcriptional regulators, such as CtsR and HrcA have also been shown to take part in the stress response in *L. monocytogenes* (Nair et al., 2000; Chaturongakul & Boor, 2004; Hu et al., 2007a, 2007b; Chaturongakul et al., 2011; Guariglia-Oropeza et al., 2014; Liu et al., 2019).

Alternative sigma factors

Sigma factors are bacterial transcription initiation factors that enable specific binding of RNA polymerase to gene promoters. Differential association between alternative sigma factors and core RNA polymerase allows the RNA polymerase to recognize specific promoter sequences and initiate transcription of targeted genes under specific conditions. *L. monocytogenes* contains up to four alternative sigma factors (σ^B, σ^C, σ^H, and σ^L) depending on the strain, in addition to the housekeeping sigma factor RpoD (σ^D). The four alternative sigma factors regulate transcription of genes important for virulence and for response to various stress and growth conditions (Glaser et al., 2001; Chaturongakul et al., 2008; Chaturongakul et al., 2011). The alternative sigma factors involved in temperature, osmotic, oxidative, or pH stresses in *L. monocytogenes* are presented in Table 4.

Table 4. Alternative sigma factors of *Listeria monocytogenes* involved in temperature, osmotic, oxidative, or pH stresses

Factor	Stresses	Reference
Alternative sigma factors		
σ^B	Cold, acid, alkali, osmotic, oxidative	Wiedmann et al. 1998; Chan et al., 2007a, 2008; Chaturongakul et al. 2011; Liu et al. 2019; Cortes et al., 2020
σ^C	Cold, heat	Zhang et al., 2005; Chan et al., 2008
σ^H	Cold, acid, alkali	Rea et al., 2004; Chan et al., 2008; Liu et al., 2016
σ^L	Cold, acid, osmotic	Okada et al., 2006; Chan et al., 2008; Raimann et al., 2009
Regulators of σ^B		
RsbT, RsbV	Cold, acid, osmotic, oxidative	Chaturongakul & Boor, 2004; Chan et al., 2008; Zhang et al., 2013

Alternative sigma factor σ^B controls the general stress response (GSR) in *L. monocytogenes* (Kazmierczak et al., 2003; Chaturongakul and Boor, 2006; Abram et al., 2008). Among the alternative sigma factors in *L. monocytogenes*, σ^B has the largest regulon (Chaturongakul et al., 2011). The main role of σ^B is to regulate the expression of various genes related to the stress reponse in *L. monocytogenes*. The stress response mechanisms are triggered by activation of σ^B-dependent promoters (Van Schaik and Abee, 2005).

Wiedmann et al. (1998) were the first to recognize the role of σ^B in acid tolerance and virulence of *L. monocytogenes*. In *L. monocytogenes,* σ^B has been shown to control the mechanisms involved in tolerance to acid stress, alkali stress, osmotic stress, cold and freezing stress, oxidative stress, and high hydrostatic pressure (Becker et al., 2000; Ferreira et al., 2001; Fraser et al., 2003; Chaturongakul & Boor, 2004; Moorhead & Dykes, 2004; Wemekamp-Kamphuis et al., 2004b; Chan et al., 2007a; 2008; van der Veen et al., 2007; Abram et al., 2008; Giotis et al., 2008a; Raimann et al., 2009; Chaturongakul et al., 2011). In a literature review by Liu et al. (2019), 304 genes encoding different functions, including stress response, metabolism, and virulence, were identified to be σ^B-dependent in *L. monocytogenes*. Altogether 73 of the 304 genes were involved in different aspects of stress response (including osmotic, oxidative, acid, alkaline, and bile stress), or in survival via antibiotic resistance (Liu et al., 2019).

The σ^B regulon consists of 18 genes identified with known or putative roles in the osmotic stress response (Becker et al., 1998; Sue et al., 2004; Liu et al., 2019). These genes include σ^B-dependent osmotic stress resistance systems such as OpuC (*opuCABCD*), Gbu (*gbuABC*), and BetL (*betL*). These systems are needed for accumulation of compatible solutes under osmotic stress conditions (Sleator et al., 1999; Fraser et al., 2003). Additionally, two σ^B

regulon members (*hfq, dtpT*) have been reported to be involved in *L. monocytogenes'* osmotic stress resistance as well as other stress responses. Hfq is a regulator binding to small RNAs, and Hfq deletion mutants have shown diminished resistance to osmotic and ethanol stresses (Christiansen et al., 2004) whereas the di- and tripeptide transporter dtpT plays a role in salt-stress protection (Wouters et al., 2005).

The σ^B regulon includes 14 members with known or putative roles in the oxidative stress response (Bucur et al., 2018; Liu et al., 2019). These genes include superoxide dismutase (Sod) encoding gene *LMRG 00891*, as well as SpxA encoding gene *LMRG 01641*. Sod protects organisms against superoxides and reactive oxygen species (ROS) and SpxA is required for oxidative stress response (Archambaud et al., 2006).

A total of 12 genes have been shown to be part of the σ^B regulon related to acid stress response. *L. monocytogenes* uses the glutamate decarboxylase (GAD) system to withstand acid stress (Liu et al., 2019). Among genes encoding components of the GAD system, *gadB, gadC,* and *gadD* belong to the σ^B regulon. The GAD system is needed to reduce acidification inside the cell (Cotter et al., 2001; Wemekamp-Kamphuis et al., 2004b). Another important mechanism activated by σ^B under acid stress involves putative arginine deiminase genes, *arcA, arcB* and *arcD*, which are regulated by σ^B-dependent putative regulator ArgR (Ryan et al., 2009).

The σ^B regulon also includes: six members involved, or putatively involved, in antibiotic resistance; three members involved, or putatively involved, in bile stress response; and 24 members involved, or putatively involved, in other stress responses (Liu et al., 2019). The regulon includes two genes involved in alkali stress, one involved in cold stress, and 21 with roles in general stress response (Liu et al., 2019). σ^B has been reported to be necessary for *L. monocytogenes* to grow at low temperatures. A σ^B-dependent gene, *ltrC*, is essential for *L. monocytogenes* to grow at cold, (Chan et al., 2007a). Among the 24 σ^B-dependent general stress response genes are *htrA, fri,* and the *clpC*. HtrA is a serine protease required for the optimal growth of *L. monocytogenes* under salt, heat and oxidative stress (Wonderling et al., 2004), Fri promotes tolerance to oxidative stress (Olsen et al., 2005), and the clpC operon is involved in heat and osmotic stress (Rouquette et al., 1996).

The transcription of *sigB* and the SigB regulatory genes (*rsb*) are under a σ^B-dependent positive-feedback-loop regulation in the σ^B operon. σ^B is activated when the anti-sigma factor RsbW binds to anti-anti-sigma factor RsbV, and σ^B is released from the anti-sigma factor to initiate the transcription (Ferreira et al., 2004; Quereda et al., 2013; Guldimann et al., 2016; Liu et al., 2019). The *rsbT* and *rsbV* mutants have also shown impaired growth at low temperature as well as under acid, osmotic, and oxidative stress (Chaturongakul & Boor, 2004; Chan et al., 2008; Zhang et al., 2013).

σ^B is not the only alternative sigma factor that has been shown to play a role in the stress tolerance of *L. monocytogenes*. Other alternative sigma factors σ^C, σ^H, and σ^L (RpoN) are also important for growth at low

temperatures (Chan et al., 2008). Furthermore, deletion of σ^L impairs acid, osmotic, and ethanol tolerance (Okada et al., 2006; Raimann et al., 2009). Interestingly, several flagellar, motility, and chemotaxis genes are also regulated by σ^L (Arous et al., 2004; Chaturongakul et al., 2011). σ^H also plays a role in cold, acidic, and alkaline stress conditions and during growth in minimal media (Rea et al., 2004; Chan et al., 2008; Chaturongakul et al., 2011) and σ^C has been reported to regulate genes required in heat stress response (Zhang et al., 2005).

Some of the genes needed in the stress reponses of *L. monocytogenes* are regulated by more than one sigma factor. Overlaps have been reported between σ^B and σ^H, σ^B and σ^L, and σ^C and σ^B (Chaturongakul et al., 2011).

Two-component systems

Two-component regulatory systems (TCS) help organisms to sense and respond to changes in different environmental conditions, and operate as a stimulus-response coupling mechanism. These systems consist of a membrane-embedded histidine kinase (HK) that senses a specific environmental stimulus and a corresponding intracellular response regulator (RR) that facilitates cellular responses through transcription of the target genes (Stock et al., 2000). TCSs are important not only when bacteria encounter environmental stresses, but also under antimicrobial stress and during host infection (Beier and Gross, 2006; Fernández et al., 2010).

Signal transduction is accomplished in TCSs through the phosphorylation of response regulators by histidine kinases. Histidine kinases are transmembrane proteins that contain a histidine phosphotransfer domain and an ATP binding domain (Mascher et al., 2006). Response regulators are usually multi-domain proteins that function as DNA-binding factors (West and Stock, 2001). When sensing changes in the extracellular environment, HK initiates an autophosphorylation reaction where a phosphoryl group is transferred from adenosine triphosphate (ATP) to a specific histidine residue. The cognate RR then catalyzes the transfer of the phosphoryl group to an aspartate residue on the receiver domain of the RR. This leads to activation of the effector domain of the RR and further on to the appropriate cellular response to the stimulus (Casino et al., 2010).

The level of phosphorylation of the RR controls its activity and the output response. The RRs are able to dephosphorylate by their own autophosphatase activity, and many HKs are also bifunctional and are able to dephosphorylate the RRs (Stock et al., 1989; Weiss et al., 2002). Signal transduction can also happen between noncognate HKs and RRs. Some HKs, like CheA, are able to phosphorylate several RRs, and some RRs are phosphorylated by several HKs (Laub and Goulian, 2007, Goulian, 2010; Kirby, 2009).

To date, 16 TCSs have been identified in *L. monocytogenes* EGD-e strain (Glaser et al., 2001). Altogether 15 of these are complete TCSs consisting of HK and RR, whereas DegU is an orphan RR lacking a linked HK (Williams et al., 2005a). Several TCSs, as well as individual HKs and RRs, play a role in the

stress tolerance of *L. monocytogenes*. LisRK has been reported to be involved in temperature, acid, osmotic, ethanol, and oxidative stresses as well as in the resistance to cell-wall-acting antibiotics, like cephalosporins (Cotter et al., 1999, 2002; Kallipolitis & Ingmer, 2001; Sleator & Hill, 2005; Williams et al., 2005a; Chan et al., 2008; Pöntinen et al., 2015). CesRK has been shown to affect the growth under heat, osmotic, and ethanol stresses, as well as to play a role in antibiotic resistance (Kallipolitis & Ingmer, 2001; Kallipolitis et al., 2003; Williams et al., 2005a; Gottschalk et al., 2008) and AgrCA play a role under osmotic and oxidative stress tolerance (Pöntinen et al., 2017b).

Pöntinen et al. (2017b) reported that the growth of a *liaS* deletion mutant was impaired under heat, acid, alkali, osmotic, ethanol, and oxidative stress conditions, with the most notable decrease under heat and osmotic stresses. LiaSR has also been linked to nisin resistance (Cotter et al., 2002). RR KdpE plays a role in heat and osmotic stress tolerance (Kallipolitis & Ingmer, 2001; Brøndsted et al., 2003) and PhoRP, ResDE, VirRS, have been linked to temperature and ethanol stress tolerance (Williams et al., 2005a; Chan et al., 2008; Pöntinen et al., 2015, 2017b). In studies by Pöntinen at al. (2015, 2017b) the *ΔvirS* strain showed nearly completely restricted growth at high temperature and impaired growth in ethanol, and the *ΔresE* mutant showed a lower growth rate at 3°C compared to the wild-type strain.

The orphan RR, DegU, has been linked to heat and ethanol stress tolerance, and is also involved in the temperature-dependent regulation of flagellar synthesis and motility-related genes, as well as in biofilm formation and virulence (Knudsen et al., 2004; Williams et al., 2005b; Gueriri et al., 2008).

DEAD-box RNA helicases

DEAD-box proteins are conserved RNA helicases that can be found in most living organisms. They are needed in RNA metabolism and other metabolic processes (Silverman et al., 2003; Cordin et al., 2006; Linder & Jankowsky, 2011). DEAD-box proteins have been linked with cold stress tolerance in *L. monocytogenes* and many other bacteria (Charollais et al., 2004; Hunger et al., 2006; Azizoglu & Kathariou, 2010; Pandiani et al., 2010; Palonen et al., 2012; Söderholm et al., 2015), as well as with heat, alkali, and oxidative stress tolerance in *Bacillus cereus* (Pandiani et al., 2011). Markkula et al. (2012) also showed that two putative DEAD-box RNA helicase genes (*lmo0866* and *lmo1450*) of *L. monocytogenes* EGD-e were related to growth under high temperature, alkali, oxidative and ethanol stress.

Netterling et al. (2012) reported that the RNA helicase Lmo1722 was required for optimal growth at low temperatures, as well as for motility of *L. monocytogenes*. Söderholm et al. (2015) reported similar findings with RNA helicase csdA (*cbo2802*) in *C. botulinum*. RNA helicases also regulate motility in other bacteria (Xu et al., 2013; Granato et al., 2016).

Flagella- and motility related genes

Induction of flagella- and motility-associated genes in *L. monocytogenes* is temperature dependent. *L. monocytogenes* strains are highly flagellated and motile at temperatures below 30°C but they are not usually motile and lack flagella at temperatures at 37°C and above (Peel et al., 1988). The reason behind the induction of flagellation at low temperatures is not totally clear, but the biofilm formation associated with motility might be an adaptation strategy against cold stress. Another explanation could be the urge to move towards more suitable conditions in terms of nutrients or other requirements compatible with growth at low temperatures (Tasara & Stephan, 2006). Liu et al. (2002) reported up-regulation of flagella- and chemotaxis-related genes in *L. monocytogenes* in response to cold stress. Several studies since have confirmed this finding (Chan et al., 2007b; Santos et al., 2019; Won et al., 2020). The relation of chemotaxis and cold growth has also been reported for other foodborne pathogens. For instance, Palonen et al. (2011) showed that a *cheA* deletion mutant strain of *Yersinia pseudotuberculosis* showed impaired growth at low temperature.

Other regulators and stress related genes

CtsR and HrcA are transcriptional repressors that regulate transcription of several genes under different stress conditions, especially under heat shock (Karatzas et al., 2003; Hu et al., 2007a, 2007b). CtsR negatively regulates class III stress-response genes, and HrcA class I stress-response genes. On top of the heat resistance, CtsR also plays a role in acid, osmotic and oxidative stress, and HrcA in biofilm formation (Nair et al., 2000; Karatzas & Bennik, 2002; Karatzas et al., 2003; Hu et al., 2007a, 2007b; van der Veen & Abee, 2010). CtsR and HrcA also regulate several stress-related genes together with σB and the major virulence-gene regulator PrfA (Hu et al., 2007a, 2007b; Ollinger et al., 2009; Toledo-Arana et al., 2009; Chaturongakul et al., 2011).

Approximately a third of *L. monocytogenes* strains carry plasmids (Lebrun et al., 1992; McLauchlin et al., 1997). Even though the majority of the stress-response related genes are chromosomal, plasmids also carry genes related to survival under different stress conditions, such as oxidative stress (Liang et al., 2016). The presence of plasmids has also been linked to survival under acid stress, at low and high temperatures and high osmolarity (Hingston et al., 2017; Pöntinen et al., 2017a).

3 AIMS OF THE STUDY

The objective of this work was to identify and investigate the genetic factors and their roles in stress tolerance of *L. monocytogenes*.

The specific aims were to examine:

1. the genome-wide expression profiles of *L. monocytogenes ΔsigB*, *ΔsigL,* and *ΔsigBL* strains at 3°C and 37°C (I, II)

2. the role of alternative sigma factors SigB and SigL in the cold, acid, and ethanol stress tolerance of *L. monocytogenes* (I, II)

3. the role of putative DEAD-box RNA helicase –encoding genes *lmo0866*, *lmo1246*, *lmo1450,* and *lmo1722* in the cold stress tolerance of *L. monocytogenes* (III)

4. the role of the flagellar- and motility-related genes *flhA* and *motA* in the cold stress tolerance of *L. monocytogenes* (IV)

5. the putative connection between flagella formation and motility, and cold stress tolerance of *L. monocytogenes* (I–IV)

4 MATERIALS AND METHODS

4.1 BACTERIAL STRAINS, PLASMIDS AND GROWTH CONDITIONS (I-IV)

The sequenced *L. monocytogenes* strain EGD-e (Glaser at al., 2001) was used for genetic modifications and as a wild-type control strain. The strains and plasmids used are presented in Table 5.

Table 5. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Reference or source
Bacterial strains		
Listeria monocytogenes		
EGD-e	Wild-type serotype 1/2a strain	Glaser et al., 2001
ΔsigB	726 bp in-frame deletion of the *sigB* gene	I
ΔsigBL	726 and 1290 bp in-frame deletion of the *sigB* and *sigL* genes	I
ΔsigL	1290 bp in-frame deletion of the *sigL* gene	II
Δlmo0866	Deletion of *lmo0866* cds with seven upstream and 170 downstream nucleotides	III
Δ*lmo1246*	Deletion of EGD-e *lmo1246* cds with ten upstream and one downstream nucleotides	III
Δ*lmo1450*	Deletion of EGD-e *lmo1450* cds with three downstream nucleotides	III
Δ*lmo1722*	Deletion of EGD-e *lmo1722* cds	III
Δ*lmo0866*c	Δ*lmo0866*, tRNA ^{Arg}::p*lmo0866*c, complemented strain	III
Δ*lmo1450*c	Δ*lmo1450*, tRNA ^{Arg}::p*lmo1450*c, complemented strain	III
Δ*lmo1722*c	Δ*lmo1722*, tRNA ^{Arg}::p*lmo1722*c, complemented strain	III
ΔflhA	1274 bp out-of-frame deletion of the *flhA* gene	IV
ΔmotA	472 bp out-of-frame deletion of the *motA* gene	IV
Escherichia coli		
TOP10	Electrocompetent strain	Invitrogen
NEB5α	Electrocompetent strain	New England Biolabs
HB101	Conjugation donor containing helper plasmid pRK24	CRBIP[a]
XL-1 Blue	Wild-type laboratory strain for routine plasmid propagation and cloning applications	Bullock et al., 1987
Plasmids		
pMAD	Cloning plasmid for gene replacement in Gram-positive bacteria	Arnaud et al., 2004
pPL2	Site-specific integration vector	Lauer et al., 2002
pKSV7	Temperature-sensitive Gram-positive bacteria integrational vector	Smith & Youngman, 1992

[a] CRBIP, Biological Resource Centre of Institut Pasteur

L. monocytogenes strains were grown at 37°C on blood or brain heart infusion (BHI) agar plates (BD, Franklin Lakes, NJ, USA) or BHI broth (BD) supplemented with antibiotics (Sigma Aldrich, St. Louis, MO, USA) when appropriate. *E. coli* strains were grown at 37°C on Luria-Bertani (LB) agar plates or in LB broth (Difco Laboratories, Detroit, MI, USA), and were supplemented with antibiotics (Sigma Aldrich) as necessary.

The cold stress responses of wild-type EGD-e and *ΔsigB*, *ΔsigL*, and *ΔsigBL* (I and II) mutants were studied in BHI and in DM (Premaratne et al., 1991) at 4°C. To study the acid stress responses, BHI broth was supplemented with 2.5% of lactic acid, 0.35% of acetic acid or 0.35% of citric acid and pH levels were adjusted to 6.0, 5.5, and 5.5, respectively (Michel et al., 2011). Ethanol stress was studied by the addition of 99.5% ethanol to a final concentration of 5% (v/v) into DM. The strains were grown at 4°C (acid stress) or at 37°C (ethanol stress) (I, II). Cold stress responses of the EGD-e wild-type and *ΔflhA*, *ΔmotA*, *Δlmo0866*, *Δlmo1246*, *Δlmo1450*, and *Δlmo1722* mutants were studied in BHI cultures grown at 3°C and 25°C (III, IV).

4.2 GENETIC MANIPULATION OF *L. MONOCYTOGENES*

4.2.1 CONSTRUCTION OF MUTANT STRAINS (I–IV)

A homologous recombination method with pKSV7 plasmid (Smith and Youngman, 1992) was used to construct deletion mutant strains *ΔsigB*, *ΔsigL*, and *ΔsigBL* (I and II), and an allelic replacement method with pMAD plasmid (Arnaud et al., 2004) was used to construct deletion mutant strains *ΔflhA*, *ΔmotA*, *Δlmo0866*, *Δlmo1246*, *Δlmo1450*, and *Δlmo1722* (III and IV) in *L. monocytogenes* EGD-e. Splicing-by-overlap extension PCR (I–III) or restriction ligation (IV) protocols were used to to create deletions of targeted genes. Deletions were confirmed by PCR and sequencing.

4.2.2 COMPLEMENTATION OF DELETION MUTATIONS (III)

Complementation (Lauer et al., 2002) of *Δlmo0866*, *Δlmo1450,* and *Δlmo1722* mutants was performed by restoring the wild-type copy of the deleted gene into the respective deletion mutant strain using vector pPL2. The coding sequences and applicable upstream regions including the putative promoters of each gene were PCR amplified and ligated to pPL2 and then transformed into the recipient *L. monocytogenes* strains by conjugation (Ma et al., 2011). Selection of the strains carrying the pPL2 constructs was achieved with chloramphenicol treatment and confirmed by PCR. Integration of the pPL2 constructs into the chromosome was confirmed by PCR.

4.3 TRANSCRIPTIONAL ANALYSIS

4.3.1 TOTAL RNA ISOLATION (I–IV)

The EGD-e wild-type strain and the mutant strains (I–IV) were grown in BHI broth as three individual cultures and sampled at the mid-exponential growth phase at 3°C (I–IV), 25°C (III, IV), and 37°C (I–IV). The specific time point for sampling was determined from the growth curves. For the cold shock experiment (III), samples grown to mid-exponential growth phase at 37°C were cooled rapidly to 5°C in an ice bath and sampled 30 min, 3 h, and 7 h after the cold shock. Total RNA was isolated with Qiagen RNeasy Midi-kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, with the following exceptions. The cells were lysed in TE buffer (Fluka Biochemica, Buchs, Switzerland) containing lysozyme 25 mg/ml and mutanolysin 250 U/ml (Sigma-Aldrich) and a DNase treatment was performed twice: an on-column treatment with Qiagen RNase-Free DNase set (Qiagen), and a second one after isolation with Ambion DNA-free kit (Ambion, Austin, TX, USA). The RNA yield was determined by using a Nanodrop ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA), and the integrity of the RNA was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

4.3.2 REVERSE TRANSCRIPTION (I–IV)

For the microarray analysis (I, II), 2 µg of total RNA of each sample was reverse transcribed into cDNA and simultaneously labeled with fluorescent dyes (Cy3 or Cy5, GE Healthcare, Little Chalfont, United Kingdom) using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) with the following modifications. The following was added to each 30 µl labeling reaction: 0.2 µg/µl random hexamers, 0.01 M DTT, 1.3 U/µl ribonuclease inhibitor, 0.5 µM dATP, dTTP and dGTP, 0.2 µM dCTP, 1.7 nmol of Cy3 or Cy5-labelled dCTP, 13 U/µl of Reverse Transcriptase (RT) enzyme, and appropriate buffer. The tubes were incubated at 46°C for 3 h and the RNA hydrolysis and inactivation of the reaction was done by adding 0.5 mM EDTA and 15 µl of 0.1 M NaOH into the reaction. After incubating the tubes again at 70°C for 15 min, the reactions were then neutralized with 15 µl of 0.1 M HCl. The labeled cDNA was purified with QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol, and the concentration of the purified product was measured with Nanodrop.

For the RT-qPCR analysis, a total of 800 ng (I, II, IV) or 400 ng (III) of total RNA of each sample was reverse-transcribed into cDNA performed using the Finnzymes Dynamo cDNA synthesis kit (Finnzymes, Espoo, Finland) according to the manufacturer's protocol, with the following exceptions. To reduce secondary structures of the template RNA, a predenaturation step (5 min at 65°C) was performed, and the incubation time and temperature of the

cDNA synthesis step were adjusted to 40 min and 40°C, respectively, to achieve sufficient RT despite possible secondary structures of the template and also for the sufficient reverse transcription of the rare target transcripts. For each individual sample of total RNA, two parallel RT reactions were performed, and also one reaction without the reverse transcriptase enzyme (no-RT control).

4.3.3 MICROARRAY ANALYSIS (I, II)

The in situ-synthesized Custom Gene Expression DNA-Microarrays (8 x 15 K, Agilent Technologies, Santa Clara, CA, USA) were used for the microarray analysis. The array probes were designed to represent each protein-coding sequence of two 1/2a-strains, EGD-e (Glaser et al., 2001) and F6854 (Nelson et al., 2004), and two 4b-strains, F2365 and H7858 (Nelson et al. 2004). In each array, there were at least two probes (60-mers) for each gene. A total of 300 ng of Cy-labeled cDNA from each sample was hybridized onto microarrays against an equal amount of Cy-labeled control cDNA using Agilent Gene Expression Hybridization Kit (Agilent Technologies) according to manufacturer's instructions. Altogether three biological replicates of each strain grown at either 3˚C or 37˚C were hybridized onto arrays, one of replicates with a dye swap. After 16 hours hybridization at 65°C, the arrays were washed according to the manufacturer's protocol (Gene Expression Wash Buffer Kit, Agilent Technologies), and scanned with an Axon GenePix 4200AL Microarray Scanner (Molecular Devices, CA, USA). Image analysis was done with GenePix Pro 6.0 software (Molecular devices). Quantile-normalization was applied to background correct the intensities. Differentially expressed genes were recognized using moderated t-test (eBayes) with False Discovery Rate (FDR) corrected P-values (limma package in R, eBayes). The gene transcripts displaying ≥2.5-fold (equivalent to ±1.3 $\log_2$) change in expression with a moderated t-test statistical significance of P-value ≤0.01 were considered differentially expressed. Differentially expressed genes were divided into different functional categories based on annotations provided by the Comprehensive Microbiological Resource of the J. Craig Venter Institute (CMR-JVCI). The microarray data have been stored in the NCBI's Gene Expression Omnibus (Edgar et al., 2002; GEO accession number GSE32434).

4.3.4 QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION PCR (RT-QPCR) (I–IV)

The Rotor-Gene 3000 instrument (Corbett Research, Sydney, Australia) was used for the real-time PCR reactions with two technical replicates for each cDNA sample. The cDNA samples were diluted 1:10, and the real-time qPCR was performed using SYBR Green chemistry (Dynamo Flash SYBR Green qPCR kit, Finnzymes) accordance with the manufacturer's instructions. A melting-curve analysis was performed at the end of each run to ensure that no

non-specific products were amplified. 16S *rrn* (I–IV) and *gap* (IV) were used as reference genes (Tasara and Stephan, 2007) and the transcript levels of the genes of interest were normalized to the transcript levels of 16S *rrn* and *gap*. For the 16S *rrn* analysis, samples were diluted 1:1000 (I, II, IV) or 1:5000 (III). Amplification efficiencies were determined for each primer pair. The relative expression levels of genes of interest at 3°C, 25°C, and 37°C were calculated using the comparative Cq method ($2^{-\Delta\Delta Ct}$ method) (Schmittgen and Livak, 2008). The relative gene expression data from quantitative real-time PCR were analyzed using Student's t-test and differences with p-values <0.05 considered significant.

4.4 CHARACTERIZATION OF GENETICALLY MODIFIED *L. MONOCYTOGENES*

4.4.1 GROWTH CURVE ANALYSIS (I–IV)

Inoculums for testing the stress tolerance of the *ΔsigB*, *ΔsigL*, and *ΔsigBL* (I, II) were prepared from frozen stocks by streaking the strains onto blood agar and incubating plates overnight at 37°C. Single colonies were picked from the plates and inoculated into 10 ml of BHI broth. The strains were grown to stationary growth phase (approximately 10^9 CFU/ml) by incubating the tubes for 16 h at 37°C with shaking (150 rpm). The bacterial cultures were then used to inoculate either normal or modified BHI or DM broths at approximately 10^3 CFU/ml and the cultures were incubated at 4°C without agitation or at 37°C with 150 rpm. Growth of the strains was monitored by viable cell counts as previously described (Loepfe et al., 2010) with four independent experiments and two replicates per experiment. The lag phases and growth rates were calculated using the DMFit program (version 2.1). The statistical significances between the mutant strains and the wild-type strain in growth parameters were calculated using Student's t-test where p-values <0.05 were considered significant.

For growth-curve analysis, the EGD-e wild-type and mutant strains *Δlmo0866*, *Δlmo1246*, *Δlmo1450* and *Δlmo1722* (III), as well as *ΔflhA* and *ΔmotA* (IV), were grown on blood agar plates and three individual colonies of each strain were picked from overnight culture and inoculated into 10 ml of BHI broth. Cultures were grown with shaking (200 rpm) for 16–17 h at 37°C and diluted 1:100 with fresh or control growth media. The optical density at 600 nm (OD600) was monitored every 15 min for 24 hours (25°C and 37°C) or once per hour for 21 days (3°C) with a Bioscreen C automated optical density monitoring system (Labsystems, Helsinki, Finland) in microtiter plates (300 µl per well) with three replicates per culture. The OD_{600} data were fitted to growth curves using the nonlinear least squares function in statistical computing package R (Venables and Ripley, 2002) to obtain the growth rates and maximum OD_{600} levels for each strain (I), or with DMFit program (version

2.1) (Baranyi & Roberts, 1994) (II). Differences in growth rates and maximum optical densities between each mutant and the wild-type strain were tested by Student's t-test and differences with p-values <0.05 were considered significant.

4.4.2 PHENOTYPIC MICROARRAY ANALYSIS (I, II)

The analysis of the phenotypic traits of the EGD-e wild-type and the mutant strains *ΔsigB, ΔsigL,* and *ΔsigBL* was performed using BIOLOG (Bochner et al., 2001) phenotypic microarrays (PMs) according to the manufacturer's protocol. The strains were tested on PM panels PM01 and PM02 to assess growth on different carbon sources, PM9–10 to test growth under osmotic, ionic, and pH stress conditions, and PM11–20 to evaluate chemical sensitivity profiles of the strains.

4.4.3 MOTILITY ASSAYS (I–IV)

Swarming motility of the wild-type deletion mutants and complementation strains (I–IV) was determined by transferring 5 µl of an overnight BHI culture of each strain onto surface of trypticase soy broth (Difco Laboratories) solidified with 0.25% agar (Kathariou et al., 1995). The plates were incubated at 3°C for eight (III), six (I, II), or three (IV) weeks, at 10°C (III) for one week and at 25°C (III, IV) and 37°C (I–IV) for 24 h.

The motility of the EGD-e wild-type strain and the mutant strains *ΔflhA* and *ΔmotA* (IV) was also examined by phase-contrast microscope (Olympus BX51, Olympus, Tokyo, Japan) at 100-fold magnification using a hanging-drop preparation of a liquid cultures grown at 3°C, 25°C, and 37°C to mid-logarithmic growth phase. The type of motility of the bacteria was evaluated qualitatively either as as smooth swimming or tumbling.

4.4.4 ELECTRON MICROSCOPY (I, IV)

The presence of flagella in EGD-e wild-type and the mutant strains Δ*sigB*, Δ*sigL*, Δ*sigBL,* Δ*flhA,* and Δ*motA* was examined using Jeol 1200 EX II (IV) (Jeol Ltd, Tokyo, Japan) or Tecnai 12 (I) (Philips Electron Optics, Holland) transmission electron microscopes at the Electron Microscopy Unit of the Institute of Biotechnology, University of Helsinki. Cells from 1 ml of BHI cultures grown to mid-logarithmic growth phase at 3°C (I, IV), 25°C (IV) and 37°C (I, IV) were washed with physiological saline (IV) or fixed with 5% glutaraldehyde (Sigma-Aldrich, St. Louis, MO, USA), incubated at room temperature for 2 hours and washed with autoclaved water (I). The cells were then prepared on carbon-coated grids and negatively stained with 3% uranyl acetate (IV) or 1% phosphotungstic acid hydrate (I) (Sigma-Aldrich).

4.4.5 CORRESPONDENCE BETWEEN VIABLE CELL NUMBERS AND OD_{600} READINGS (III, IV)

The correspondence between the viable cell numbers and OD_{600} readings of the wild-type EGD-e and the mutant strains grown in BHI broth at 3°C and 37°C were examined at three (III) or two (IV) different time points representing early logarithmic, late logarithmic, and early stationary (III), or early logarithmic and mid-logarithmic (IV) growth phases with standard colony-counting method using plate count agar (Difco laboratories).

4.4.6 MINIMUM GROWTH TEMPERATURES (III)

The mean minimum growth temperatures of the wild-type and *Δlmo0866*, *Δlmo1246*, *Δlmo1450,* and *Δlmo1722* mutant strains were examined with five replicate cultures per strain as described by Hinderink et al. (2009) using the Gradiplate W10 temperature gradient incubator (BCDE Group, Helsinki, Finland) with the following modifications. Diluted cultures grown for 20 hours in BHI broth were plated by stamping technique onto tryptic soy agar (TSA) (BD). The strains were grown in the Gradiplate incubator for 21 days with a temperature gradient from 1.0°C to 9.5°C and from 8.7°C to 16.8°C. The minimum growth boundaries were observed with a stereomicroscope and the temperature at which dense growth stopped and separate colonies appeared was determined as the minimum growth temperature.

5 RESULTS

5.1 TRANSCRIPTIONAL ANALYSES

5.1.1 GENOMEWIDE TRANSCRIPTIONAL ANALYSIS OF *ΔSIGB*, *ΔSIGL,* AND *ΔSIGBL* AT 3°C AND 37°C (I, II)

Transcriptome analysis using cultures grown in BHI to mid-logarithmic phase at 3°C revealed: 198 and 127 genes that were down- and up-regulated, respectively, in *ΔsigB* strain; 237 and 159 genes that were down- and up-regulated, respectively, in *ΔsigL* strain; and 254 and 198 genes that were down- and up-regulated, respectively, in *ΔsigBL* strain, compared to the EGD-e wild-type. At 37°C, 86 and 39 genes were down- and up-regulated, respectively, in *ΔsigB* strain; 203 and 108 genes were down- and up-regulated, respectively, in *ΔsigL* strain; and 139 and 90 genes were down- and up-regulated, respectively, in *ΔsigBL* strain, compared to the EGD-e wild-type. In *ΔsigB* strain, 29 down-regulated genes were present at both 3°C and 37°C. In *ΔsigL* strain, 47 down-regulated genes were present at both 3°C and 37°C. In *ΔsigBL* strain 63 genes were down-regulated at both 3°C and 37°C. An overview showing the distribution of down-regulated genes at 3°C and 37°C is shown in Figure 1.

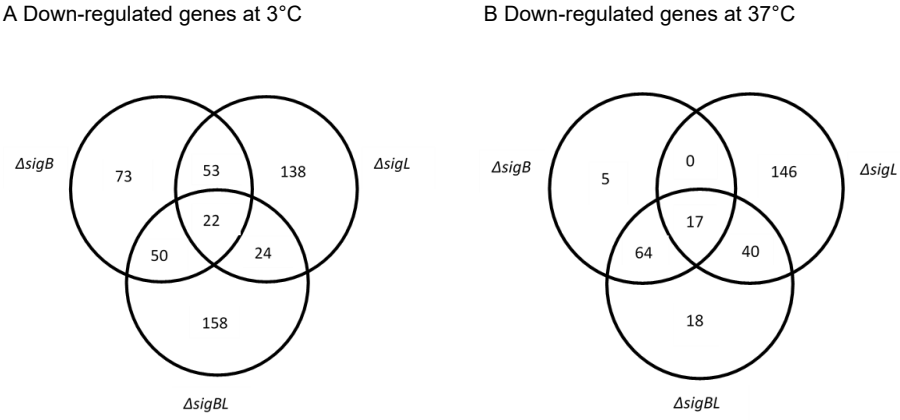

Figure 1 Overview of down-regulated genes in *Listeria monocytogenes ΔsigB*, *ΔsigL*, and *ΔsigB*L compared to the wild-type EGD-e strain detected during exponential growth in BHI at 3°C (A) and 37°C (B). Genes showing 2.5-fold change (P-value ≤ 0.01) in transcript abundance were considered differentially expressed. Adopted from I and II.

The genes down-regulated in *sigB*, *sigL,* and *sigBL* null mutants were assigned into fifteen functional categories based on putative or confirmed functions of their protein products (I,II). The majority of the genes down-regulated in *ΔsigB* relative to the wild-type strain during exponential growth in BHI at 3°C were involved in transcription regulation (12%), cellular processes (11%), energy metabolism (9%), and viral-associated functions (6%). Among the down-regulated genes in *ΔsigB* at 37°C relative to wild-type strain transport and binding (12%), cellular processes (9%), energy metabolism (9%), and amino-acid biosynthesis (7%) categories had relatively the largest percentages of down-regulated genes.

The categories overrepresented with down-regulated genes in *ΔsigL* during exponential growth in BHI at 3°C relative to the wild-type strain were cellular processes (11%), energy metabolism (11%), transport and binding (8%), regulatory (6%) and viral functions (6%). At 37°C the categories with the largest percentages of down-regulated genes were protein synthesis (24%), energy metabolism (10%), transport and binding (9%) and viral functions (5%).

The majority of down-regulated genes in *ΔsigBL* during exponential growth in BHI at 3°C relative to the wild-type strain belonged to the groups of transport and binding proteins (13%), protein synthesis (13%), energy metabolism (8%), and cellular processes (7%). At 37°C, the categories with the largest percentages of down-regulated genes were protein synthesis (15%), transport and binding proteins (11%), energy metabolism (8%), and cellular processes (8%).

Genes encoding proteins that are part of the ABC transport system and related to the phosphotransferase system (PTS), as well as fructose- and mannose-specific genes, were found to be down-regulated in all three mutants at both temperatures. Genes encoding ribosomal genes were strongly down-regulated in *ΔsigL* at 37°C and also in *ΔsigBL* at 3°C and 37°C, but not in *ΔsigB*. A large group of chemotaxis and motility-related genes were found to be down-regulated in *ΔsigL* at 3°C but only a few of these genes were down-regulated in *ΔsigB* or *ΔsigBL*. Bacteriophage-A118–associated protein-encoding genes were again strongly down-regulated in *ΔsigL* at 3°C and 37°C, and also in *ΔsigB* at 3°C, but not in *ΔsigBL* at either temperature.

5.1.2 RELATIVE EXPRESSION OF DEAD-BOX RNA HELICASES *LMO0866*, *LMO1246*, *LMO1450,* AND *LMO1722* AND FLAGELLAR GENES *FLHA* AND *MOTA* AT LOW TEMPERATURES (III, IV)

The relative expression levels of the DEAD-box RNA helicase genes *lmo0866, lmo1246, lmo1450,* and *lmo1722* were significantly ($p<0.05$) higher during continuous growth at 3°C than at 37°C, and also 30 min, 3 h, and 7 h after cold shock from 37°C to 5°C. The relative expression levels of *lmo0866, lmo1246,*

lmo1450, and *lmo1722* during continuous growth and after the cold shock are presented in III.

The relative transcript levels of flagellar genes *flhA* and *motA* in cultures grown in BHI to mid-logarithmic phase at 3°C were 465- and 238-fold higher than at 37°C, respectively ($p<0.01$). The relative expression of *flhA* in cultures grown at 3°C was also significantly higher (3.2-fold) than at 25°C ($p<0.01$).

5.2 CHARACTERIZATION OF *L. MONOCYTOGENES* MUTANT STRAINS (I–IV)

5.2.1 GROWTH UNDER STRESS CONDITIONS (I–IV)

The growth of the wild-type strain and mutant strains was similar in BHI at 4°C, while in DM the growth of the *ΔsigL* and *ΔsigBL* strain was significantly compromised compared with the wild-type. All three organic acids—lactic, acetic and citric acids—affected the growth of the *ΔsigL* and *ΔsigBL* strain drastically at 4°C. When exposed to acetic acid and citric acid, no detectable exponential growth was observed, however the number of viable colony-forming units was diminished directly after exposure to these acids. In the presence of lactic acid, the lag phases of *ΔsigL* and *ΔsigBL* were significantly longer and the growth rates were drastically compromised compared with the wild-type strain. In the presence of acetic acid and lactic acid, the lag phase of the *ΔsigB* mutant was significantly longer relative to the wild-type strain while the growth rates of these two strains were approximately equal. When the strains were grown in DM supplemented with ethanol at 37°C, the lag phases were significantly longer and the growth rates were significantly compromised in *ΔsigB, ΔsigL,* and *ΔsigBL* relative to the wild-type strain. Without ethanol supplementation, the growth of the mutants and the wild-type strain in DM at 37°C was similar (Figure 3).

Growth of the *Δlmo0866* and *Δlmo1722* was totally impaired in BHI at 3°C and in *Δlmo1450* only a slight growth was detected. Growth of the *Δlmo1246* was also impaired at 3°C compared with the wild-type strain. Cold-temperature growth was restored in the complemented *Δlmo0866*c, *Δlmo1450*c, and *Δlmo1722*c strains to a similar level as in the wild-type strain. The growth of the *Δlmo1246* and the wild-type strain was identical at 25°C and 37°C while the growth rates and maximum optical densities of the *Δlmo0866, Δlmo1450,* and *Δlmo1722* strains were significantly smaller than in the wild-type strain also at these temperatures (Table 6). The minimum growth temperatures of the *Δlmo0866*, *Δlmo1450,* and *Δlmo1722* strains were 5.1°C, 4.9°C and 8.8°C higher, respectively, compared to the wild-type EGD-e strain after 21 days incubation in a temperature gradient incubator.

Deletion in *flhA* and *motA* significantly decreased the growth rates and the maximum optical densities when grown in BHI at 3°C (Table 6). At 37°C, the growth of *ΔflhA*, *ΔmotA,* and the wild type strain was similar.

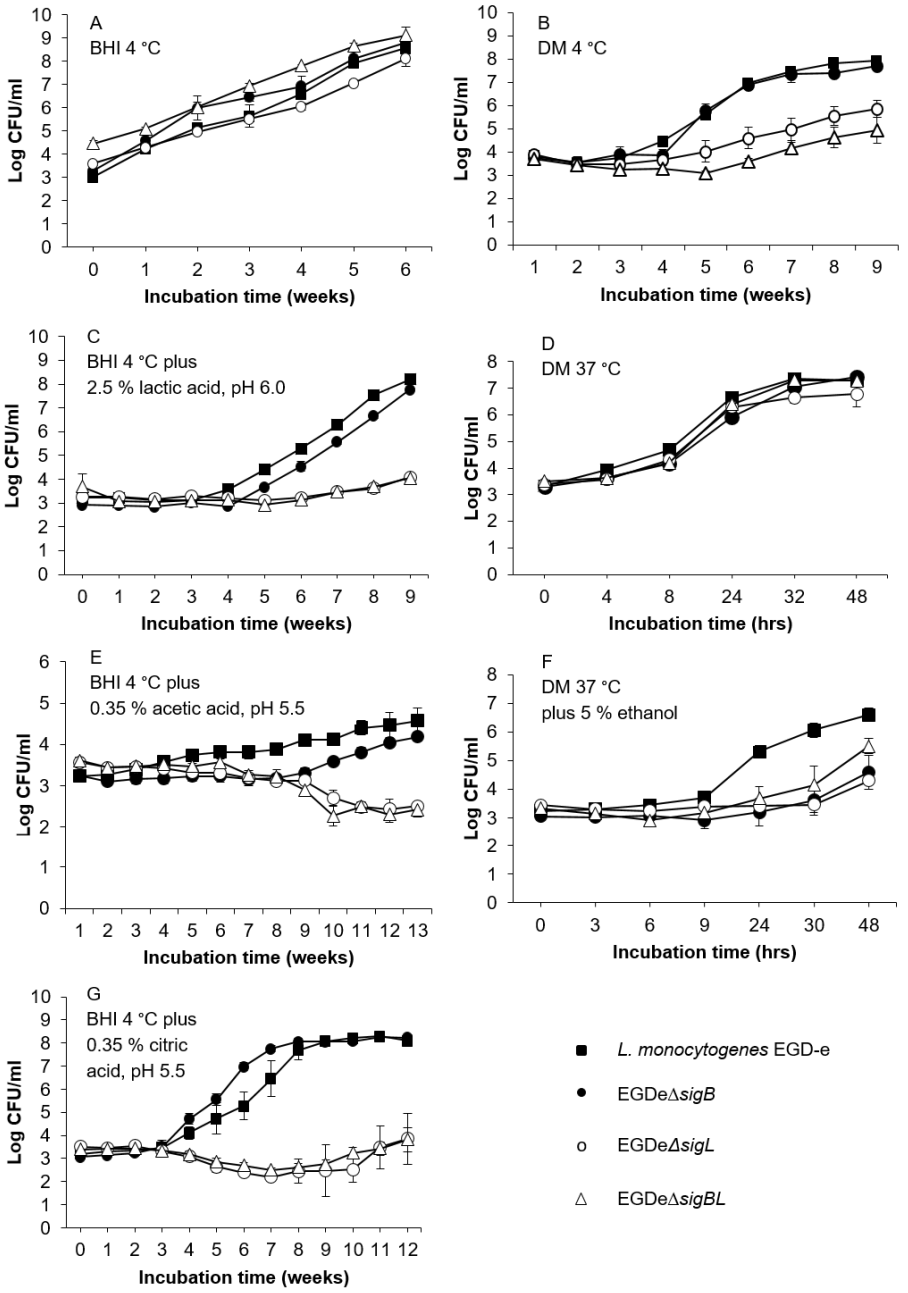

Figure 2 Growth of *Listeria monocytogenes* EGD-e wild-type strain and EGD-eΔ*sigB,* EGD-eΔ*sigL,* and EGD-eΔ*sigBL* mutant strains in BHI broth at 4°C (A), in DM at 4°C (B), in BHI supplemented with lactic acid at 4°C (C), in DM at 37°C (D), in BHI supplemented with acetic acid at 4°C (E), in DM supplemented with ethanol at 37°C (F) and in BHI supplemented with citric acid at 4°C (G). The means and standard deviations derived from four independent experiments are presented. Adopted from I and II.

Table 6. Growth parameters determined for *Listeria monocytogenes* EGD-e and the deletion mutants Δ*lmo0866,* Δ*lmo1246,* Δ*lmo1450,* Δ*lmo1722, ΔflhA* and *ΔmotA* at 3°C, 25°C and 37°C. Adopted from III and IV.

	3 °C		25 °		37 °C	
	Growth rate ± SD (OD600 units/hour)	Max. optical density ± SD (OD600 units)	Growth rate ± SD (OD600 units/hour)	Max. optical density ± SD (OD600 units)	Growth rate ±SD (OD600 units/hour)	Max. optical density ± SD (OD600 units)
EGD-e	0.12±0.009	0.72±0.014	0.19±0.003	0.96±0.002	0.29±0.004	0.75±0.013
Δ*lmo0866*	0.00±0.002*	0.08±0.030*	0.10±0.008*	0.90±0.022*	0.24±0.006*	0.73±0.011
Δ*lmo1246*	0.09±0.003*	0.62±0.025*	0.17±0.018	0.95±0.027	0.28±0.007	0.73±0.004
Δ*lmo1450*	0.01±0.001*	0.15±0.027*	0.07±0.004*	0.87±0.013*	0.17±0.004*	0.78±0.021
Δ*lmo1722*	0.00±0.000*	0.06±0.008*	0.12±0.004*	0.89±0.003*	0.26±0.006*	0.75±0.017
	Growth rate ± SD (OD600 units/hour)	Max. optical density ± SD (OD600 units)	Growth rate ± SD (OD600 units/hour)	Max. optical density ± SD (OD600 units)	Growth rate ± SD (OD600 units/hour)	Max. optical density ± SD (OD600 units)
EGD-e	0.04±0.002	1.18±0.03	0.34±0.001	1.53±0.00	0.47±0.007	1.46±0.01
Δ*flhA*	0.03±0.001*	0.85±0.01*	0.34±0.02	1.54±0.01	0.45±0.02	1.44±0.01
Δ*motA*	0.03±0.002*	0.92±0.00*	0.34±0.01	1.54±0.01	0.46±0.03	1.45±0.00

*Significant difference (Student's t-test, P<0.001) compared with the corresponding value of the wild-type EGD-e.

5.2.2 PHENOTYPIC MICROARRAYS (I, II)

The growth of the wild-type strain and the *sigB* null mutant was similar on different carbon sources (PM01 and PM02) while the *sigL* and *sigBL* null mutant displayed significantly slower growth compared to the wild-type strain on PM01 in the presence of N-acetyl-D-glucosamine as a carbon source. The *ΔsigBL* strain showed slower growth also on PM02 with 3-0-b-D-galactopyranosyl-D-arabinose as a carbon source. When exposed to osmotic or low pH stress conditions (PM09-PM10), the *sigB* null mutant and the parental strain again showed similar growth, while the *ΔsigL* displayed significantly slower growth in the presence of 5% sodium lactate, 7% urea, and in pH 5 compared to the wild-type strain. The *ΔsigBL* strain showed significantly slower growth in the presence of 3% sodium formate, 7% urea, and 100 mM sodium nitrite compared to the wild-type strain. In the *ΔsigB* strain, only seven chemical compounds on PM11–20 impaired growth compared to the wild-type strain (I) while 121 chemical compounds impaired the growth of *ΔsigL* 35 and *ΔsigBL* (I, II). Relative to the wild-type strain, the *sigB* null mutant showed increased sensitivity to compounds targeting DNA synthesis and protein synthesis, as well as to toxic ions, while the *ΔsigL* and *ΔsigBL* mutants displayed increased sensitivity to protein-synthesis inhibitors, cell-wall-synthesis–targeting antibiotics, and to compounds targeting DNA and RNA metabolism, as well as respiration inhibitors, toxic ions, and detergents.

5.2.3 FLAGELLA FORMATION (I, IV)

Electron microscopy revealed that the wild-type EGD-e strain and *ΔsigB* and *ΔsigBL* mutants formed flagella at 3°C, whereas the *ΔsigL* strain was nonflagellated. The number of flagella in the *ΔsigBL* strain was nevertheless smaller at 3°C than in the wild-type. The EGD-e strain and the *ΔmotA* mutant strains formed flagella at 25°C. However, the number of flagella in *ΔmotA* was notably smaller than in the wild-type. The *ΔmotA* strain appeared nonflagellated at 3°C, whereas the wild-type EGD-e strain was flagellated. The *ΔflhA* strain appeared nonflagellated at all the temperatures tested. At 37°C, none of the strains formed flagella.

5.2.4 MOTILITY (I–IV)

At 3°C, the wild-type, *ΔsigB,* and *ΔsigBL* strains all showed growth zones of a similar shape and size around the inoculation spots whereas the *ΔsigL* mutant strain was non-motile. The wild-type EGD-e strain and *Δlmo1246* showed similar swarming pattern at 25°C on semi-solid agar, whereas the diameter of the swarm ring around the inoculation point of the *Δlmo1722* was approximately half relative to the wild-type strain. Because of the poor growth of *Δlmo1722* at 3°C, the motility of this strain could not be tested. The

Δlmo0866, Δlmo1450, ΔflhA, and *ΔmotA* mutant strains were non-motile at all the tested temperatures. At 37°C, the wild-type EGD-e strain and all the mutant strains were non-motile.

6 DISCUSSION

6.1 TRANSCRIPTIONAL ANALYSIS OF *ΔSIGB*, *ΔSIGL*, AND *ΔSIGBL* AT LOW AND OPTIMAL GROWTH TEMPERATURE AND THE ROLE OF THE ALTERNATIVE SIGMA FACTORS SIGB AND SIGL IN THE STRESS TOLERANCE OF *L. MONOCYTOGENES* (I, II)

Alternative sigma factors are known to be important transcription regulators of stress tolerance and adaptation in *L. monocytogenes*. They regulate various cellular processes needed for growth and survival under unfavourable conditions (Becker et. al, 1998; Wiedmann et al., 1998; Becker et. al, 2000; Kazmierczak et al., 2003, Wemekamp-Kamphuis et al., 2004b; van Schaik and Abee, 2005; Okada et al., 2006; Chan et al., 2007a; Marles-Wright & Lewis, 2007; Chaturongakul et al., 2008; Raengpradub et al., 2008; Raimann et al., 2009; Oliver et. al, 2010; Gorski et. al, 2011; Liu et. al, 2017; Liu et. al, 2019; Guerreiro et al., 2020). To gather more profound information about the regulatory role of sigma factors at low temperatures, this thesis work investigated the regulons of σ^B and σ^L, and the expression profile of the *ΔsigBL* double-mutant strain with a whole-genome expression analysis at 3°C and 37°C.

A whole genome expression analysis revealed 198 genes positively regulated by σ^B during exponential growth at 3°C. At 37°C there were 86 genes positively regulated by σ^B. In total 29 genes were found to be under positive σ^B transcriptional regulation during exponential growth at both 3°C and 37°C. These included genes coding for PTS system components, universal stress-protein family members, pyruvate metabolism proteins and internalin H. In previous studies the σ^B regulon has been assessed in different growth conditions. Kazmierczak et al. (2003) identified 55 and Raengpradub et al. (2008) 168 genes positively regulated by σ^B in *L. monocytogenes* strain 10403S at stationary growth phase or under salt stress. In a study by Hain et al. (2008) the EGD-e strain was grown at 37°C and 105 genes were found to be positively regulated by σ^B. Oliver et al. (2010) suggested that the σ^B core regulon consists of at least 63 genes using lineage I, II, IIIA, and IIIB strains and Mujahid et al. (2013) recognized 15 σ^B dependent genes when comparing the genes or proteins positively regulated by σ^B using microarrays, proteomic studies and RNA-sequencing. The σ^B regulon of *L. monocytogenes* has recently been extensively reviewed by Liu et al. (2019). They described 73 σ^B regulon members that are involved in different aspects of stress response and survival, including osmotic, oxidative, acid, alkaline, and bile stress, or are involved in antibiotic resistance.

Strong similarities were detected between previous studies and this study's data, especially at 37°C. Nevertheless, a considerable number of σ^B-dependent genes detected during cold growth in this study were not identified in these former reports. Interestingly, genes *lmo1041–lmo1048* coding proteins needed in molybdenum transport and molybdenum and molybdopterin biosynthesis were found here to be positively regulated through σ^B during *L. monocytogenes* growth at 3°C. In *E. coli,* the transcription of moa locus, which encodes enzymes required for molybdopterin biosynthesis, is known to be controlled at two sigma-70-type promoters immediately upstream of the *moaA* gene. The expression of the *moa* locus was also shown to be enhanced under anaerobiosis (Andersson et al., 2000). Although further experiments are still needed, our findings suggest that genes *lmo1041–lmo1048* may play a part in the cold stress response of *L. monocytogenes*. Whether the expression of these genes is enhanced under other environmental stress conditions in *L. monocytogenes* also warrants further investigation.

At 3°C, 237 genes were detected to be under positive σ^L dependent transcriptional control, and at 37°C the number of down-regulated genes was 203. Only 47 of these genes exhibited positive σ^L transcriptional regulation at both temperatures. Apart from four genes, all of these 47 genes were located within the bacteriophage A118 harboring region in the genome of *L. monocytogenes* EGD-e.

Several genes encoded by the bacteriophage A118 locus, as well as proteins with prophage function from the EGD-e genome, such as the putative Phage shock protein C gene, were all found to display positive σ^L transcriptional dependency. Similar positive σ^{54} (σ^L) transcriptional dependency of phage genes, as well as bacterial-host–associated phage shock genes, have been documented in other bacteria (Weiner et al., 1991; Ceyssens et al., 2008). Based on the current transcriptome observations, it may be predicted that the replication ability of the A118 bacteriophage in σ^L lacking *L. monocytogenes* EGD-e cells will be reduced compared to the parental strain. This however remains to be examined, and similarly the implications of these observations in view of the ability of other bacteriophages to replicate in *L. monocytogenes* cells warrants further examination. For example, temperature-dependent phage resistance, via currently unknown molecular mechanisms, has been observed in *L. monocytogenes* epidemic clone II strains (Kim and Kathariou, 2009). Thus, it would be interesting to determine whether σ^L might be contributing to the molecular mechanisms involved in phage resistance. In our study, an operon consisting of bacteriophage-A118–associated protein encoding genes *lmo2278–lmo2301* was down-regulated in *ΔsigB* strain grown at 3°C but not at 37°C. Repression of these genes was lost at both 3°C and 37°C in the *sigBL* null mutant background.

Of the 254 genes down-regulated in *ΔsigBL* at 3°C, 38% were also detected in *sigB* or *sigL* single gene deletion mutants and 62% were present only in *ΔsigBL*. Similarly, at 37°C 87% of the 139 down-regulated genes were present in either *ΔsigB* or *ΔsigL* and the remaining 13% were detected only in *ΔsigBL*.

It thus appears that growth at low temperature expands the specialized expression profile of *ΔsigBL,* but at optimal growth temperature the *ΔsigBL* expression profile resembles more the expression profiles of *ΔsigB* and *ΔsigL*.

Genes *lmo1152–lmo1167* in putative propanediol utilization (*pdu*) operon responsible for the catabolism of 1,2-propanediol were amongst the genes that were down-regulated only in *ΔsigBL,* but not in *ΔsigB* or *ΔsigL* during exponential growth at 3°C. Recent studies have suggested that the Pdu cluster can support the growth of this pathogen in specific conditions along the food chain (Tang et al., 2015; Zeng et al., 2019). Liu et al. (2019) also suggested that the pdu operon is part of the σ^B regulon.

Genes *lmo1983–lmo1991* of the predicted *ilv-leu* operon encoding IlvD, IlvB, IlvH, IlvC, LeuA, LeuB, LeuC, LeuD, and IlvA, known to be involved in the synthesis of branched-chain amino acids, valine, isoleucine, and leucine, were all down-regulated in *ΔsigBL* at 3°C but not in *ΔsigB* or *ΔsigL* at 3°C or at 37°C in any of the mutant strains. Garmyn et al. (2012) previously compared the *L. monocytogenes* EGD-e transcriptomes at 25°C and 37°C and found that genes in both *pdu* and *ilv-leu* operons were up-regulated at 25°C compared to 37°C. Tojo et al. (2004) also reported down-regulation of the *ilv-leu* operon during logarithmic-phase growth under nitrogen-limited conditions in *Bacillus subtilis*. Consequently, the expression of the *ilv-leu* operon genes seem to be linked to unfavourable growth conditions. Marinho et al. (2019) described recently that the σ^B-dependent regulatory sRNA Rli47 represses isoleucine biosynthesis in *L. monocytogenes* through a direct interaction with the *ilvA* transcript.

Furthermore, all but 3 of the 12 genes in the operon comprising purine ribonucleotide biosynthesis genes (*purD, purG, purN, purM, purF, purQ, purl, lmo1771, purC, purB, purK,* and *purE)* were repressed in *ΔsigBL* at 3°C, but not at 37°C or in *ΔsigB* or *ΔsigL*. Liu et al. (2017) reported recently that the operon (LMRG_00978–LMRG_00986) which encodes proteins involved in pyrimidine ribonucleotide biosynthesis is regulated by σ^B. Our findings support the role of alternative sigma factors as the regulators for nucleotide metabolism.

Operon-encoding PTS-system related and fructose- and mannose-specific genes *lmo0398–lmo0402* were found to be down-regulated in *ΔsigBL* at 3°C, but not at 37°C. The same operon has been previously reported to be co-regulated through multiple transcription factors, including σ^B and σ^L (Abram et al., 2008; Chaturongakul et al., 2011). Palmer et. al (2009) studied the contributions of σ^B and σ^L to *L. monocytogenes* response to antimicrobial substance, nisin, and to transcription of putative bacteriocin immunity gene *lmo2570*. The gene *lmo2570* was found to be regulated by σ^B but not by σ^L. In our study the gene *lmo2570* was also down-regulated in *ΔsigB* and *ΔsigBL* mutants at 37°C, but not in *ΔsigL* mutant.

The phenotypic evaluation of the *ΔsigB*, *ΔsigL,* and *ΔsigBL* mutants revealed impaired growth in the presence of organic acids and ethanol at low growth temperature. The growth of the *sigB* null mutant was compromised in

the presence of lactic acid, acetic acid, and ethanol, while both the *sigL* and *sigBL* null mutants showed impaired growth in the presence of lactic acid, acetic acid, citric acid, and ethanol, relative to the wild-type strain. The growth of the *sigL* and *sigBL* null mutants was also slower at low temperature (4°C) in DM relative to the parental strain. In addition, phenotypic microarray analysis exposed significant differences in growth of the EGD-e wild-type strain and the *sigB*, *sigL,* and *sigBL* null mutant strains on different carbon sources and in the presence of various chemical compounds. Overall, the growth of the *ΔsigBL* mutant in acid, ethanol, and temperature stresses resembled more the phenotype of the *ΔsigL* than the phenotype of *ΔsigB*. These findings support the previous findings of the role of SigB in the stress response *of L. monocytogenes* against acid and low temperature stress (Wiedmann et al., 1998; Ferreira et al., 2001; Moorhead et al., 2003; Wemekamp-Kamphuis et al., 2004b; Liu et al., 2019; Cortes et al., 2020). Raimann et al. (2009) has also previously reported impaired growth of *sigL* null mutant under cold, organic acid, and elevated NaCl salt concentration stress conditions.

Phenotypic microarray analysis revealed 24 compounds affecting the growth of the *sigBL* null mutant strain, but not that of *ΔsigB* or *ΔsigL*. Most of these chemical compounds were antibiotics targeting cell wall, membrane, DNA, or protein synthesis. Apparently the deletion in both *sigB* and sigL increases the sensitivity of the *L. monocytogenes* EGD-e towards different stress conditions and chemical compounds and brings out phenotypic characteristics not present in *sigB* or *sigL* null mutant strains. The stress response mechanisms positively regulated by both *sigB* and *sigL* might thus be more relevant than it has been previously suspected.

6.2 THE ROLE OF DEAD-BOX RNA HELICASES AND FLAGELLAR GENES *FLHA* AND *MOTA* IN COLD TOLERANCE OF *L. MONOCYTOGENES* (III, IV)

Transcriptomic and phenotypic analyses of DEAD-box RNA helicase encoding gene *lmo0866, lmo1246, lmo1450,* and *lmo1722* deletion mutants were performed at 3°C, 25°C, and 37°C. The relative expression levels of *lmo0866, lmo1246, lmo1450,* and *lmo1722* were significantly higher during continuous growth at 3°C than at 37°C, and also 30 min, 3 h and 7 h after cold shock from 37°C to 5°C. Similarly, the expression of the counterparts of *L. monocytogenes* DEAD-box RNA helicases in *B. cereus* showed higher transcript levels at 10°C than at 37°C (Pandiani et al., 2010). Chan et al. (2007a) reported elevated expression levels of *lmo0086*, *lmo1450,* and *lmo1722* homologues of *L. monocytogenes* 10403S at low growth temperature. On the other hand, Cabrita et al. (2015) reported that persistent *L. monocytogenes* isolates showed significantly lower levels of *lmo1722*, and *lmo0866* genes under cold growth compared to the sporadic strains.

Three out of four of these mutants, *Δlmo0866*, *Δlmo1450,* and *Δlmo1722* showed strong inhibition in growth at 3°C. The growth of *Δlmo0866* and *Δlmo1722* was totally impaired, and *Δlmo1450* showed only slight growth at 3°C. The minimum growth temperatures of these mutants were also significantly higher compared to the wild-type strain. Bäreclev et al. (2014) showed that *Δlmo0866*, *Δlmo1450,* and *Δlmo1722* mutant strains of *L. monocytogenes* showed impaired growth at suboptimal temperature (16°C). It thus appears that *lmo0866, lmo1450,* and *lmo1722* have a significant role in the growth of *L. monocytogenes* at low temperatures. The restored cold-temperature growth of the complemented *Δlmo0866*c, *Δlmo1450*c, and *Δlmo1722*c strains to the wild-type level support this suggestion. In previous studies (Charollais et al., 2004; Hunger et al., 2006; Pandiani et al., 2010; Palonen et al., 2012; Söderholm et al., 2015; Jiang et al., 2019) the inactivation of DEAD-box proteins has been shown to be linked to cold sensitivity in *E.coli*, *B. subtilis*, *B. cereus*, *Y. pseudotuberculosis,* and *C. botulinum*.

The fourth DEAD-box RNA-helicase-encoding gene-deletion mutant, *Δlmo1246*, showed only a slight decrease in growth at 3°C. The growth of this mutant was similar compared to the wild-type at 25°C and 37°C while the *Δlmo0866*, *Δlmo1450,* and *Δlmo1722* also showed a minor growth deficit at these temperatures. These findings suggest that *lmo1246* has no significant role in the cold tolerance of *L. monocytogenes* nor is it essential for growth at any of the temperatures tested. The study by Bäreclev et al. (2014) confirms this finding. Previous studies (Pandiani et al., 2010, 2011) with *B. cereus* DEAD-box RNA helicase CshD, similar to Lmo1246, has shown that this helicase differing from the C-terminal sequences from the other helicases has no role in the temperature tolerance. The considerable growth deficit of the *Δlmo1450* at 25°C and 37°C suggest a more universal role for *lmo1450* in *L. monocytogenes*.

The role of flagella synthesis and motility genes *flhA* and *motA* in the cold stress tolerance of *L. monocytogenes* was studied at low (3°C), suboptimal (25°C) and optimal (37°C) growth temperatures. The relative expression levels of *flhA* and *motA* were found to be higher at 3°C compared to both 25°C and 37°C. The growth of the EGD-e*ΔflhA* and EGD-e*ΔmotA* deletion mutant strains was compromised at 3°C relative to the wild-type strain. These results illustrate that *flhA* and *motA* have a role in the cold-stress tolerance of *L. monocytogenes,* yet the exact functions and regulation of these genes at low growth temperatures is still unknown. The up-regulation of flagellar genes and higher expression of flagellar proteins in *L. monocytogenes* at low growth temperatures has been reported also in other studies (Liu et al., 2002; Chan et al., 2007b; Won et al., 2020). Santos et al. (2019) also recently reported that several motility-associated proteins, including FlhA and MotA, were more abundant at 10°C and 25°C compared to optimal growth temperature, suggesting that there is an association between these genes and the cold stress tolerance of *L. monocytogenes*.

6.3 ASSOCIATION BETWEEN FLAGELLA FORMATION AND MOTILITY, AND STRESS TOLERANCE OF *L. MONOCYTOGENES* (I–IV)

Deletion mutant *ΔsigL* was completely non-motile at 3°C and 10°C, and un-flagellated at 3°C. The *ΔsigB* and *ΔsigBL* mutants showed a similar swarming pattern compared to the wild-type at 3°C, but the flagella formation of *ΔsigBL* was slightly compromised at 3°C compared to wild-type. The growth of *ΔsigL* and *ΔsigBL* was inhibited when grown in DM at 4°C, but not in BHI at the same temperature. Apparently the poor nutrient availability in addition to low growth temperature stress brought out the temperature-sensitive phenotypes of these two mutants. Nevertheless, the cold stress alone failed to trigger growth deficits of *ΔsigL* and *ΔsigBL*.

The cold-sensitive deletion mutant strains *Δlmo0866* and *Δlmo1450* were completely non-motile in BHI at 3°C, while the swarming pattern of the *Δlmo1246* was similar to the wild-type strain. The mutant strains *ΔflhA* and *ΔmotA* were completely non-motile at 3°C and 25°C, and flagella formation was deficient. The growth of these strains was also significantly compromised grown in BHI at 3°C. These findings suggest that the motility and flagella formation may play a role in the optimal cold response of *L. monocytogenes*.

The relationship between motility and stress tolerance has been discussed in previous studies (Karatzas et al., 2003; Giotis et al., 2010; Van Boeijen et al., 2010). Casey et al. (2014) showed that exposure of *L. monocytogenes* to a sub-lethal concentration of the biocide quaternary ammonium compound benzethonium chloride (BZT) significantly increased the relative expression of motility- and flagella-related genes, including *flhA* and *motA*. Guariglia-Oropeza et al. (2018) reported up-regulation of motility-related genes *in L. monocytogenes* under acid and bile stress. The chemotaxis gene *cheA* has also been shown to play a significant role in both cold growth and and motility in *Y. pseudotuberculosis* (Palonen et al., 2011). Interestingly, csp genes, particularly cspA and cspB, seem to influence flagella production and extracellular motility in *L. monocytogenes* (Eshwar et al., 2017; Kragh et al., 2020). Similar findings have also been made in *C. botulinum* (Derman et al., 2015).

7 CONCLUSIONS

1. Our whole genome expression analysis revealed 198 and 86 genes positively regulated by σ^B during exponential growth at 3°C and 37°C, respectively. Altogether 29 genes were found to be under positive σ^B transcriptional regulation during exponential growth at both temperatures. At 3°C, 237 genes were detected to be under positive σ^L-dependent transcriptional control and at 37°C the number of down-regulated genes was 203. Only 47 of these genes exhibited positive σ^L transcriptional regulation at both temperatures. Of the 254 genes down-regulated in *ΔsigBL* at 3°C, 38% were also detected in *ΔsigB* or *ΔsigL*, and 62% were present only in *ΔsigBL*. Similarly, at 37°C 87% of the 139 down-regulated genes were present in either *ΔsigB* or *ΔsigL* and the remaining 13% were detected only in *ΔsigBL*. It thus appears that growth at low temperature expands the specialized expression profile of *ΔsigBL,* but at optimal growth temperature the *ΔsigBL* expression profile resembles more the expression profiles of *ΔsigB* and *ΔsigL*.

2. The phenotypic evaluation of the *ΔsigB*, *ΔsigL,* and *ΔsigBL* mutants of *L. monocytogenes* EGD-e revealed impaired growth in the presence of ethanol, and organic acids at low growth temperature. The growth of the *sigL* and *sigBL* null mutants was also slower at low temperature (4°C) in DM relative to the parental strain. Phenotypic microarray analysis revealed several compounds affecting the growth of the *sigBL* null mutant strain that did not affect the growth of neither *ΔsigB* nor *ΔsigL*. Apparently deletion in both *sigB* and *sigL* increases the sensitivity of the *L. monocytogenes* towards different stress conditions and chemical compounds and promotes phenotypic characteristics absent from both *sigB* and *sigL* null mutant strains.

3. The relative expression levels of *lmo0866, lmo1246, lmo1450,* and *lmo1722* were significantly higher during continuous growth at 3°C than at 37°C, and also after cold shock from 37°C to 5°C. The deletion of *lmo1450* resulted in a strong inhibition of growth at 3°C, and the growth of *Δlmo0866* and *Δlmo1722* was totally impaired. The results suggest that *lmo0866, lmo1450,* and *lmo1722* play an important role in the cold stress tolerance of *L. monocytogenes* strain EGD-e.

4. The relative expression levels of *flhA* and *motA* were found to be higher at 3°C compared to both 25°C and 37°C, and the growth of the *ΔflhA* and *ΔmotA* deletion mutant strains was compromised at 3°C relative to the wild-type EGD-e strain. These results suggest that *flhA* and *motA*

are needed for the optimal growth of *L. monocytogenes* strain EGD-e at low temperatures.

5. The cold-sensitive deletion mutant strains *Δlmo0866*, *Δlmo1450, ΔflhA,* and *ΔmotA* were completely non-motile at 3°C. This suggests that the cold stress response and motility of *L. monocytogenes* might be linked.

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